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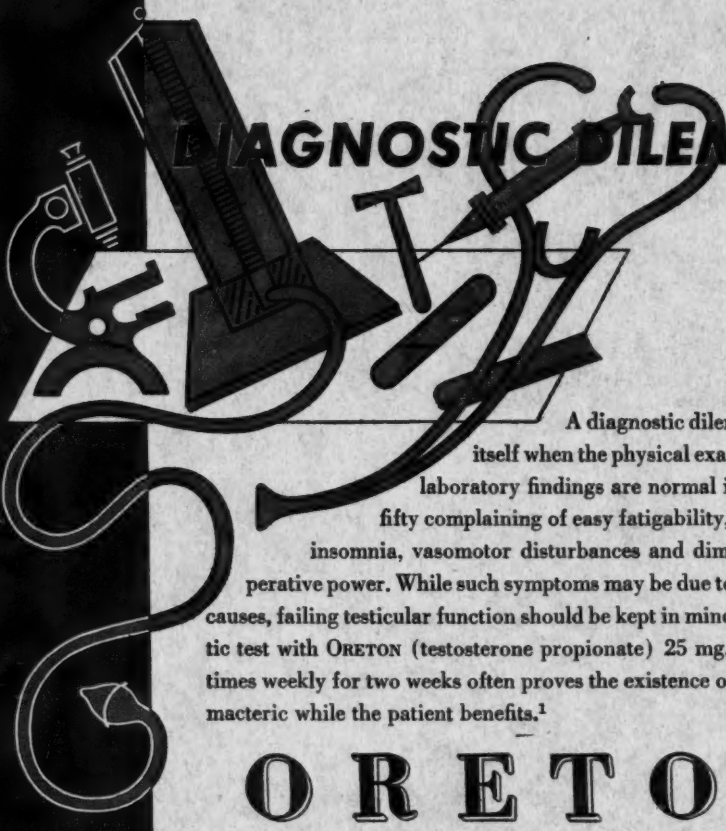

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ENDOCRINOLOGY

VOLUME 38 MAY, 1946 NUMBER 5

THE EFFECT OF THYROXIN AND DINITROPHENOL ON THE THERMAL RESPONSES TO COLD

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IT IS CLEAR from the work of Cramer and Ludford (1926), Krogh, Lindberg and Okkels (1932), Fischborn (1935), Baillif (1937), Starr and Roskelley (1940), and others that the thyroid gland enlarges and the height of its follicular cells increases when animals are exposed over long periods of time to a cold environment. In addition, Kenyon (1933) and Bernstein (1941) have noted hypertrophy and hyperplasia of the thyroid and an increased blood flow through the gland. Further, Schenk (1922) detected thyroid substance in the blood of rabbits exposed to cold. Finally Ring (1939) and Le Blond and Gross (1943) added significantly to the knowledge of the role of the thyroid gland in adjustment to low environmental temperatures by showing that the basal metabolism of normal rats exposed to cold for three weeks increased approximately twenty per cent, while the metabolism of thyroidectomized rats similarly treated did not increase (Ring, 1939). The survival of thyroidectomized rats in an environmental temperature of 0°–2°C. is prolonged by thyroxin substitution therapy (Le Blond and Gross, 1943).

The data on the physiological changes resulting from brief periods of extreme cold are far less extensive and the results equivocal. Though Kenyon (1933) reported that the thyroid gland in rats exposed to cold for six to twenty-four hours showed no activation, Ariel and Warren (1943) observed an increase in follicular cell height in the thyroids of rabbits within one-half hour after exposure of the animals to cold. Further evidence of the brief time necessary for activation was noted in the effect of cold on the thyroid gland in vitro (Turner and Turner, 1945).

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[1] Aided by the technical assistance of E. Dean and A. Cady.

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As to the thermal and metabolic changes associated with short periods of intense cold, there are reported a fall in skin temperature (Herrington and Gagge, 1943) and a rise in metabolic rate (Goldfether, 1941). That the thyroid plays a role under such circumstances is suggested by Ring (1942) who stated "the maximal catabolic response to acute cold is potentiated by the thyroid hormone."

If the protection afforded the intact animal in an acutely cold environment by the thyroid gland merely resulted from an increased cellular metabolism, then other stimulants of cell metabolism might well be expected to affort similar protection. Accordingly this study was undertaken to compare the results of two such stimulants, thyroxin and dinitrophenol, on the hypothermia of mice subjected to a low environmental temperature.

MATERIALS AND METHODS

Pure strain male albino mice, approximately 100 days of age and weighing twenty grams were used. During a preliminary control period, metabolic rate observations at 30° C. were made by use of a modified Warburg apparatus. Each vessel had a volume of approximately 500 cc. and the carbon dioxide was absorbed by 10 cc. of 25 per cent sodium hydroxide placed in the bottom of the jar. The animals rested on copper mesh screen held above the alkali by glass supports. Readings were taken every three minutes for a period of one hour and appropriate calculation of cc. O_2/gr./hr. (Q_{O_2}) made. Activity of the animals was observed through a slit in the Warburg manometer stands which permitted a view of the mice through the glass wall of the thermostatic bath.

One group of eight mice was given intramuscular injection of pure synthetic thyroxin on alternate days. The dose, 2 mgm./kilo, was given in mammalian Ringer's solution; it was based on the dosage of Boettiger (1941) for dwarf mice, and on that used by Ring (1942) for rats. The dinitrophenol injections (30 mgm./kilo) were given to a second group of eight animals immediately preceding the metabolic or body temperature determination, since the onset of the rise in metabolism following injection is very rapid (Hall, et al., 1933). To reduce the variability, this latter group served as the normal control group two weeks before the dinitrophenol injections.

The oxygen consumption of each group of animals was measured with the thermostat at 5°C. to determine the effectiveness of dinitrophenol and thyroxin in raising the metabolic rate in cold and to determine the relative calorigenic responses to these stimulants at this temperature.

In separate experiments designed to determine the effect of these stimulants in the maintenance of body temperature during acute cold, the mice were placed in vessels at 5°C. and the rectal temperature was measured at five-minute intervals for one hour by means of a potentiometer[2] and a small iron-constantan thermocouple.

OBSERVATIONS

Data on the effect of an environmental temperature of 5°C. on the mean rectal temperature of the three groups of mice are presented

[2] Leeds and Northrup Company.

TABLE 1. TIME COURSE OF MEAN RECTAL TEMPERATURE AT 5°C.

Minutes	Groups		
	Control	Thyroxin	D.N.P.
0	38.7°C.	38.6°C.	38.3°C.
10	36.8	38.2	35.3
15	36.0	37.7	34.8
20	35.9	37.1	34.0
25	35.3	35.9	33.5
30	35.3	35.7	32.9
35	34.6	35.5	32.0
40	34.2	35.3	30.9
45	34.0	35.0	30.5
50	33.4	34.7	29.9
55	33.2	34.3	29.3
60	31.9	33.6	27.5

in Table 1 and graphically represented in Figure 1. The values for the thyroxin group were obtained after eight injections of 2 mgm./kilo, and the values for the dinitrophenol (D.N.P.) group after a single 30 mgm./kilo injection. The observed differences in response between the control and dinitrophenol groups are highly significant. The P value in this case is <.0001, i.e., such a difference would be expected to occur by chance alone less than once in 10,000 times. The differences between the control and thyroxin groups are also signifi-

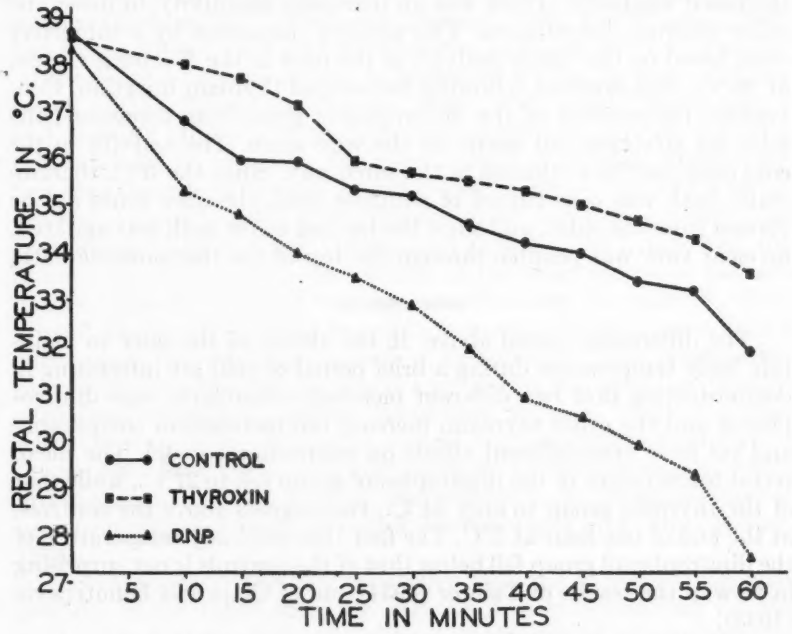

FIG. 1. Time course of mean rectal temperatures of control, thyroxin and dinitrophenol groups at 5°C.

cant. The P value is less than .001. The method used was that of Fisher (1936), p. 104.

Table 2 presents the mean oxygen consumption (Q_{O_2}) for these same groups of animals at 5°C.

In addition, the thyroxin administration was continued to include a total of twelve injections. At this time, this group of mice was placed in the jars with the thermostat at −3°C. At the same time, a normal group of mice was subjected to this lower temperature. At the end of one hour the mean rectal temperature of the thyroxin group fell to 29°C, while that of the control group fell to less than 22°C. This difference in rate of fall in body temperature would be expected

TABLE 2. MEAN Q_{O_2} VALUES AT 5°C.

Minutes	Groups		
	Control	Thyroxin	D.N.P.
0–30	7.5	8.7	8.6
30–60	4.8	8.7	5.1

to occur by chance alone less than once in 1,000,000 times (Fisher, 1936).

The activity of the thyroxin group at all times appeared to be increased markedly. There was an increased sensitivity to noise and other external disturbances. This activity, measured by a subjective scale based on the visible activity of the mice in the Warburg vessels, at 30°C., was doubled following the second thyroxin injection. Conversely, the activity of the dinitrophenol group was depressed; the mice lay stretched out prone on the wire mesh. The activity in the cold could not be evaluated in the same way. Since the 5°C. thermostatic bath was constructed of stainless steel, the mice could not be viewed from the sides, and since the ice and water bath was agitated, no clear view was possible through the top of the thermostatic bath.

DISCUSSION

The differences, noted above, in the ability of the mice to maintain body temperature during a brief period of cold are interesting in demonstrating that two different metabolic stimulants, one dinitrophenol and the other thyroxin, increase the metabolism comparably and yet have very different effects on adaptation to cold. The mean rectal temperature of the dinitrophenol group fell to 27°C., while that of the thyroxin group to only 34°C., two degrees above the controls, at the end of one hour at 5°C. The fact that the body temperature of the dinitrophenol group fell below that of the controls is not surprising in view of the results of Tainter (1934) and of Giaja and Dimitrijevic (1933).

It is clear that if, at the onset of the experiment, the heat produc-

tion is the same, the difference in body temperature may be accounted for either by a difference in heat loss of the two groups of animals and/or by a difference in ability to maintain the high metabolic rate over the hour of exposure to cold.

Evidence in favor of greater heat loss of the animals receiving dinitrophenol is the fact that the initial rectal temperature of each mouse, even before exposure to cold, was always lower than that of animals receiving thyroxin (see Figure 1). Two factors may be responsible for this increased heat loss: the decrease in vasoconstriction in cold with dinitrophenol (which, at least at room temperature, is a potent vasodilator (Hall et al. 1933) and the facilitation of heat loss by the resting of the animals upon the copper mesh in the cold environment. Herrington (1940) has pointed out that the failure of heat regulation in small animals is "not due to overcoming the heat producing capacity."

Indication of the difference in ability of the mice to maintain the high level of metabolism may be obtained from the fact that the oxygen consumption of the dinitrophenol group fell during the last half hour of adjustment to cold, while the oxygen consumption of the thyroxin group remained high (see Table 2). Such an ability to maintain the oxygen consumption may in part be due to the greater spontaneous activity of mice receiving thyroxin (see Results). The relationship of the thyroid gland to activity has received considerable attention (Richter, 1937; Hall and Lindsay, 1938; Kruskinsky, 1940; and Brody, 1941). That thyroid substance increases activity by acting on the central nervous system is suggested by the findings of Kommerell (1931), Kunde and Neville (1930), Pick (1931), and Issekutz and Issekutz (1935). The participation of thyroid secretion in acute cold adaptation, however, has been largely overlooked despite the fact that the rate of thyroid output in cold is almost doubled (Dempsey and Astwood, 1943). Nevertheless, many investigators including Sherrington (1924), Lusk (1928), and Odum (1942) indicated the importance of neuromuscular activity in the maintenance of metabolism in animals subjected to a cold environment. In addition, Ring (1942) has reported that, in cold, thyroxin exerts a potentiating effect upon the calorigenic action of epinephrine. Such potentiation may result from the effectiveness of epinephrine in raising the response of skeletal muscle to nervous stimulation (Gruber, 1922) at a time when thyroxin is increasing the central nervous system discharge to the muscles. Ring (1942) could demonstrate no such potentiation with dinitrophenol, a finding possibly attributable to the decreased spontaneous activity of animals receiving this substance (Hall and Lindsay, 1934).

There appears then the possibility that the increased muscular activity of the thyroxin group may be in part responsible for the maintenance of the high rate of heat production in an environment

where heat loss is considerable. Also, the lack of such activity in the dinitrophenol group may be responsible for the decreased heat production in the last half hour of exposure to cold as well as for the greater heat loss throughout the whole hour period suffered by reason of the large amount of ventral body surface exposed to the copper mesh.

It would thus appear from the data presented here, that a possible explanation for the differences in the degree of hypothermia between the two experimental groups of mice upon exposure to a brief period of cold may be found in the different actions of these two metabolic stimulants at low temperature. Dinitrophenol may well be an agent acting on the central nervous system (Hall, Crismon and Chamberlin, 1937) decreasing the effectiveness of the heat regulating center in defense against cold, i.e. both increasing heat loss and decreasing muscular activity. Thyroxin, on the other hand, may increase the chemical regulation of body temperature. This action of thyroxin is probably not mediated by a simple increase in cellular metabolism, but indirectly through the increase in neuromuscular activity.

SUMMARY

Thyroxin decreased the hypothermia of mice subjected for one hour to an environmental temperature of 5° C. Dinitrophenol in doses calorigenically equal to the thyroxin increased the hypothermia.

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OBSERVATIONS ON THE CHEMICAL CYTOLOGY OF THE THYROID GLAND AT DIFFERENT FUNCTIONAL STAGES[1]

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IN A PREVIOUS INVESTIGATION (Dempsey, 1944), changes in the oxidative enzymes and in the fluorescence of the colloid were correlated with different states of thyroidal activity. Since the publication of this article, an additional number of histochemical reactions has been applied successfully to other organs and tissues, and conditions have been established under which chemical characterizations may be obtained by the use of tissue stains (cf. Dempsey and Wislocki, 1946).

Methods have also been devised whereby the functional activity of the thyroid gland can be controlled almost at will. Thyroidal activity can be regulated at predictable levels by subjecting rats to different environmental temperatures (Dempsey and Astwood, 1943). The synthesis of thyroidal hormone can be blocked completely by chemotherapeutic agents, which at the same time induce a state of extreme morphological hyperplasia (MacKenzie and MacKenzie, 1943; Astwood, Sullivan, Bissell and Tyslowitz, 1943).

These considerations indicate the feasibility of determining the results of various chemical cytological reactions when applied to thyroid glands of different functional states. Accordingly, we shall present in the following sections a description of the changes which the thyroid gland undergoes during altered activity as revealed by various procedures.

MATERIAL AND METHODS

The bulk of our experimental material consists of thyroid glands from young male rats of approximately 100–150 gms. weight. These animals were from the Long-Evans strain and were maintained at 24 ± 2°C. in our colony from birth. They were fed *ad libitum* from a stock diet of Purina fox chow. A group of these animals was killed and the thyroid glands were fixed in the various ways described below. A second group was placed in a coldroom at a temperature of 1 ± 1°C. for three days. A third group was maintained for three days in a hotroom at a temperature of 30 ± 1°C. In a fourth group, the antithyroid drug 2-thiouracil was administered in the food at a concentration of 0.1 per cent for seven days.

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[1] This investigation was aided by a grant from the Committee on Endocrinology, National Research Council

At the end of the experimental period, the rats were killed with illuminating gas. A block of tissue from the throat region containing the thyroid glands, esophagus, trachea and pretracheal muscles was removed and preserved in various fixing fluids. Phosphatase was demonstrated in sections from blocks fixed in absolute acetone or 80% alcohol which had been chilled to approximately 0°C. The methods which we have used for the demonstration of phosphatase are modifications of Gomori's (1941a; 1941b) procedures. The modifications consist in the use of longer times of incubation and in the employment of several phosphorylated substrates at pH 9.0, 7.0 and 5.0. Bauer reactions were carried out on sections previously fixed in 10% formalin, Zenker's solution; Bouin's fluid; and a mixture of alcohol, formalin and picric acid. Feulgen's procedure for desoxyribonucleoproteins was applied to Zenker-fixed material. The thyroidal reticulum was demonstrated by Pap's method on specimens fixed in various alcoholic mixtures, formalin, Bouin's and Zenker's fluid. After the latter fixative, Pap's procedure produced argyrophilic granules in the follicular cells and argyrophilia of the colloid. Argyrophilia was also revealed by Bodian's protargol method, after fixation in formalin and Bodian's fixative no. 2. Basophilia was studied in Zenker-fixed sections after staining with Mallory's eosin-methylene blue procedure and after staining in dilute solutions of methylene blue buffered to a graded series of pH values. The method of Holmgren and Wilander (1940), involving staining with toluidin blue after fixation in 4% basic lead acetate, was employed to reveal the metachromasia of certain thyroidal elements.

In addition to the rat thyroid glands obtained and prepared as described above, we have examined occasional specimens from other animals. In this way we have incidentally been able to confirm most of our principal findings on the glands from one or more additional species. Such incidental observations have been made on the thyroid glands of one horse, three monkeys, five cats, and five dogs.

RESULTS

General histological considerations. The histology and staining reactions of the thyroid gland are too well known to require any extensive treatment here. However, since we shall have occasion repeatedly to refer to various tinctorial reactions of the gland, a brief statement of these staining properties and their changes during altered thyroidal activity is in order.

The intrafollicular colloid and the cytoplasm of the follicular cells are ordinarily described as acidophilic, since they stain with varying degrees of intensity with acid dyes after procedures such as hematoxylin and eosin, Azan and Masson's stain. The colloid shows great lability in its staining properties. When stained with hematoxylin and eosin, some follicles contain colloid which is much more eosinophilic than is that of others. After staining with one of the triacid stains such as Azan, the colloid from different follicles stains differentially with azocarmine or aniline blue. After Masson's mixture the colloid stains with either ponceau or light green. By subjecting animals to experimental procedures designed to increase or decrease

thyroidal activity, it can be shown that the colloid at inactive storage phases stains red with Azan and Masson, whereas in active glands the colloid stains blue or green with the respective procedures.

In the rat and some other experimental animals, there is a well-developed zonation of the thyroid gland which apparently corresponds to different degrees of activity. The peripherally located follicles exhibit flattened or narrow follicular cells and the intrafollicular colloid is intensely chromophilic, staining with the red component of the triacid stains. The morphology of these peripheral follicles is such as to suggest a state of secretory quiescence, or storage. The follicles in the central part of the gland, on the contrary, appear active. The follicular epithelium is high cuboidal or columnar and the intrafollicular colloid stains with aniline blue after Azan and with the light green component of the Masson procedure.

Although the intrafollicular colloid stains intensely with acid dyes in the triacid mixtures, it is not entirely correct to call the colloid acidophilic. With certain staining procedures employing basic dyes, the colloid also exhibits intense basophilia. Thus, when stained with eosin-methylene blue or with methylene blue alone under controlled conditions of pH, the colloid in the peripheral follicles stains intensely blue. The more centrally located colloid, although it stains more faintly, also has an affinity for the basic dye (fig. 1).

The inactive thyroid glands from animals maintained in hot environments contain larger amounts of intensely basophilic colloid than do the glands from control animals. On exposure of the animal to cold, there is a rapid diminution in the basophilic substances in the colloid (figs. 3 and 7).

The follicular epithelium also exhibits basophilic properties. In addition to the nucleus, the cytoplasm also takes on a distinct blue color when stained with eosin-methylene blue. The cytoplasmic basophilia extends throughout all parts of the cell, although it is somewhat more concentrated in the vicinity of the nucleus than in other regions of the cell. Toward the apical margin unstained spaces frequently

---

PLATE 1

The effect of ribonuclease upon the basophilia of the thyroid colloid. The four sections illustrated on this plate were prepared from Zenker-fixed thyroids and were stained identically by the eosin-methylene blue procedure. The photographs were exposed, developed and printed for identical times. All section ×200.

Fig. 1. Thyroid gland from normal control rat. The peripheral follicles (upper part of figure) are intensely basophilic.

Fig. 2. Section adjacent to that presented in figure 1, and treated identically except for digestion in a solution of crystalline ribonuclease. The basophilic reaction of the colloid has been greatly depressed.

Fig. 3. Thyroid gland from a rat maintained in a cold room. The basophila of the colloid is reduced as compared with that of the normal control shown in figure 1.

Fig. 4. Section adjacent to that presented in figure 3, and treated identically after digestion in ribonuclease. The basophila of the colloid has been abolished.

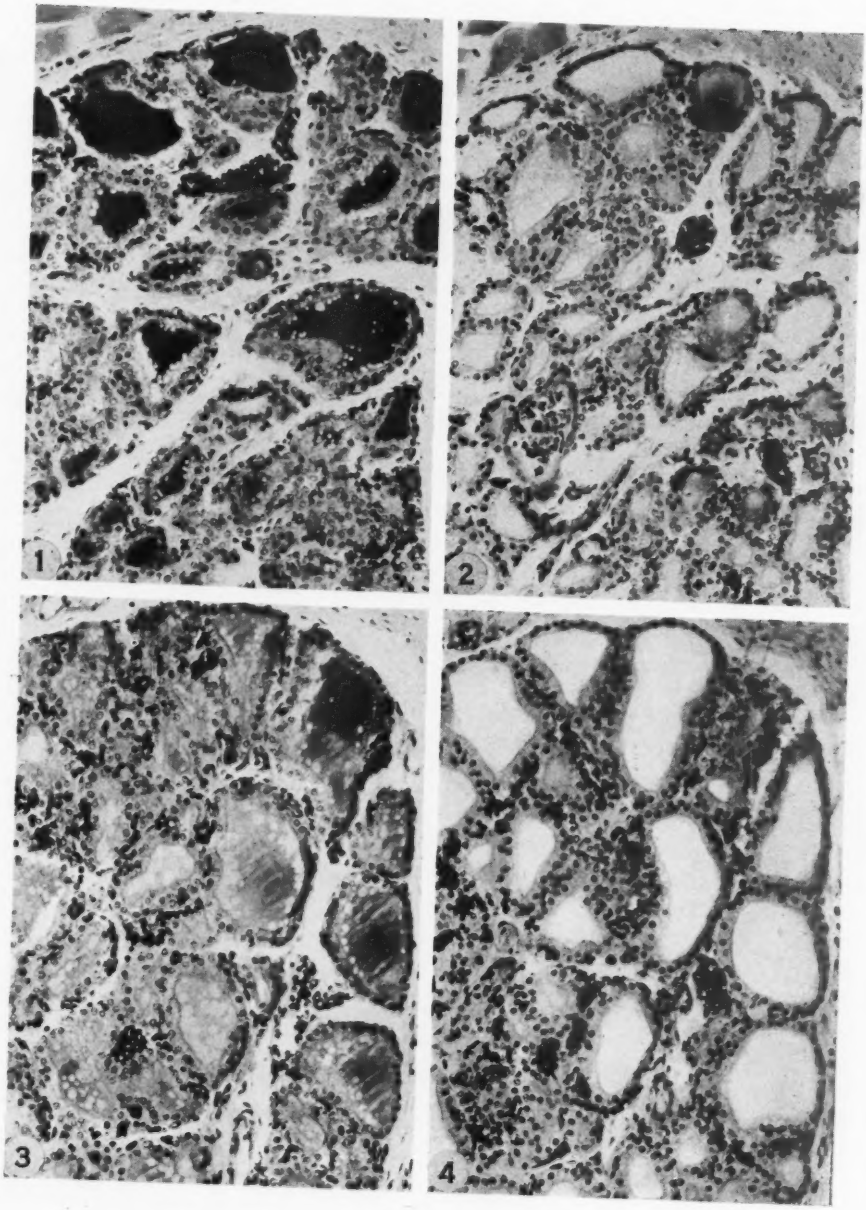


PLATE 1

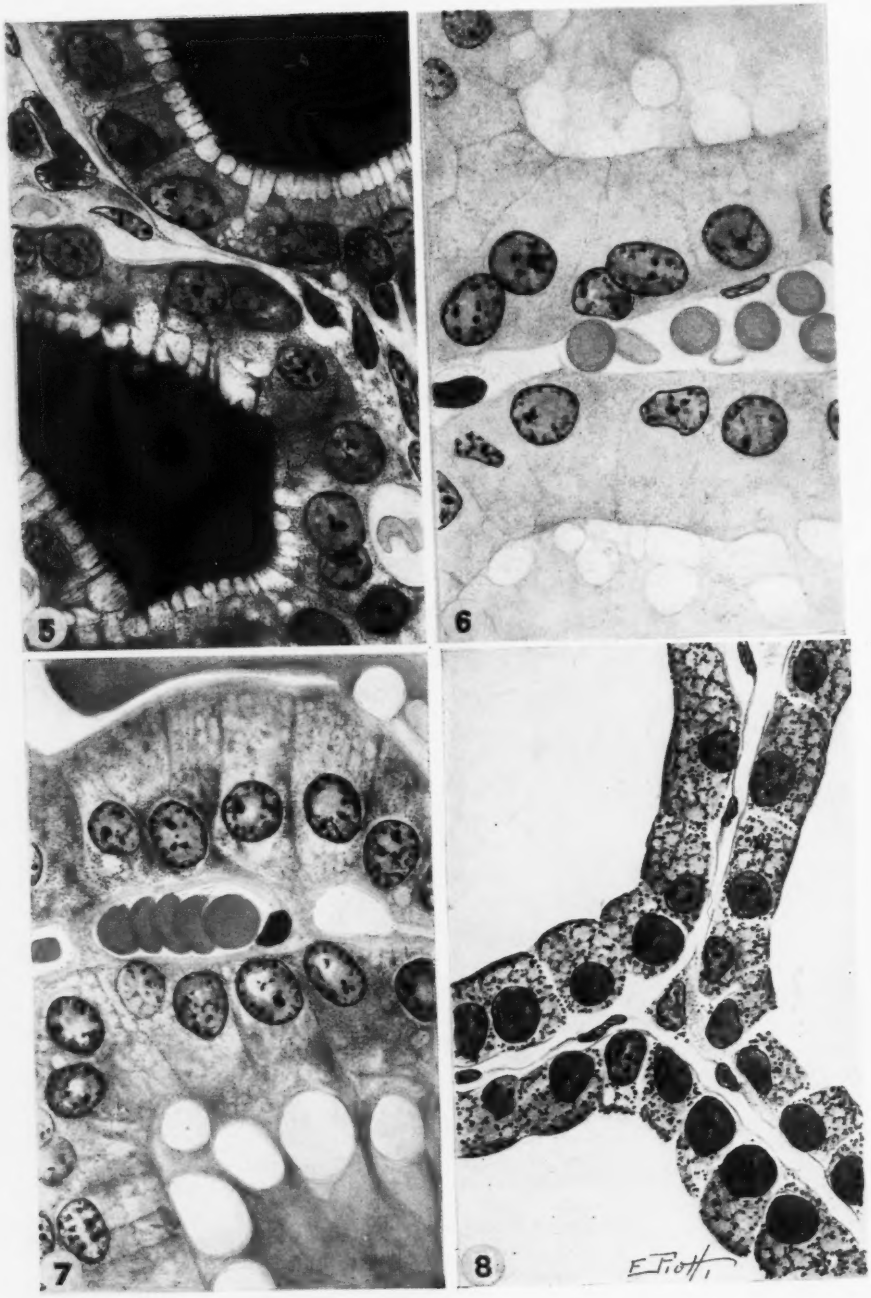


PLATE 2

occur within the cytoplasm (fig. 5). Upon activation of the thyroid gland by exposure of rats to cold environments, the cytoplasmic basophilia is somewhat reduced, presumably because the increased cellular volume leads to a dilution of the stainable substance (fig. 7).

In addition to the follicular elements described above, another thyroidal component requires mention in that it will receive occasional reference in the following sections. This is the interfollicular stroma, which consists of an exceedingly delicate argyrophilic reticulum containing among its other connective tissue cells a goodly number of scattered, usually individual, mast cells. These latter cells, interspersed as they are in the stroma, are in close relation to the thyroid capillaries which penetrate the gland and surround the follicles in a basket-like meshwork.

The basophilic elements of the thyroid gland.

1. *Feulgen reaction.* Sections of thyroid glands which had been fixed in the Zenker-acetic mixture were hydrolyzed for various lengths of time in normal hydrochloric acid at 60C. and subsequently stained in the fuchsin sulfurous acid reagent as recommended by Cowdry (1943). Optimal results were obtained after 30 and 60 minutes acid hydrolysis. The Feulgen reaction, which under these conditions is specific for thymonucleic or desoxyribonucleic acid (Stowell, 1945), gave positive results only in the nuclei of the thyroid gland. Occasional bodies exhibiting a nucleal stain were observed floating free in intrafollicular colloid. These bodies were identified as the nuclei of degenerating follicular cells which had been cast off from the epithelial walls of the follicles. The intrafollicular colloid itself, especially in the follicles at the periphery of the gland, was frequently stained with a purplish tinge; this did not represent a positive Feulgen nucleal reaction since the colloid was also stained by the Bauer procedure and by the plasmal reaction of Feulgen and Voit (1924) and therefore did not require the specific acid hydrolysis necessary for the identification of desoxyribonucleoprotein. Thus, staining of the colloid by Feul-

---

PLATE 2

Fig. 5. Drawing illustrating the basophila of the colloid and cytoplasm of the follicular cells from the thyroid gland of a normal control rat. Zenker fixation, eosin-methylene blue stain. 90× objective, 10× ocular.

Fig. 6. Drawing of a section adjacent to that shown in figure 5, illustrating the abolition of basophilia in the cytoplasm and colloid after digestion in ribonuclease. Zenker fixation, eosin-methylene blue stain. 90× objective, 10× ocular.

Fig. 7. Drawing illustrating the reduction in basophilia of the colloid and cytoplasm of the follicular cells after exposure to cold. Compare with figure 5. Zenker fixation, eosin-methylene blue stain. 90× objective, 10× ocular.

Fig. 8. Drawing illustrating argyrophilic granules in the cytoplasm of follicular cells from a dog's thyroid gland. Alcohol, formol, acetic fixation. Bodian's protargol stain. 90× objective, 10× ocular.

gen's reaction was similar to that observed by Uotila (1934), who interpreted his findings as indicating the presence of nucleoprotein in the colloid. Our results, which indicate that the specific acid hydrolysis necessary for the identification of desoxyribonucleoprotein is not required in the colloid, demand an interpretation different from that supplied by Uotila.

The nuclei of the follicular epithelium were stained somewhat more deeply in the peripheral follicles than in the central ones. A similar difference was noted in the glands from animals which had been exposed to cold. This decrease in the Feulgen reaction in active as compared with inactive thyroid follicles presumably indicates a slight decrease in the amount of nuclear desoxyribonucleoprotein in states of active secretion of the gland.

2. *Staining reactions of the thyroid gland with basic dyes.* We have already described in a previous section the affinity of both colloid and the cytoplasm of follicular cells for methylene blue when stained with the eosin-methylene blue procedure. We shall attempt, in this section, to analyze the substances responsible for these basophilic staining properties and to contrast these properties with those properties of other basophilic regions such as those of mucus, the matrix of cartilage and mast cells. Dempsey and Wislocki (1946) have recently discussed the factors responsible for staining with basic dyes, and have suggested possible methods for the differentiation of basophilic elements. Among the strongly basophilic substances are the nucleoproteins and the sulfuric acid esters present in such structures as mucus, cartilage and mast cells. These two classes of basophilic substances may be differentiated by several tests which will be described in the following paragraphs.

The sulfuric acid-protein complexes may be characterized by their metachromasia when fixed in basic lead acetate and stained by toluidin blue (Holmgren and Wilander, 1940). This procedure, when applied to our sections, reveals the mast cells, tracheal cartilage and the mucus of the tracheal epithelium by their red-violet coloration. The nuclei and the thyroid colloid, on the other hand, stain a pure blue with no reddish cast. It may be concluded, therefore, that the colloid does not contain appreciable quantities of mucoprotein.

The cytoplasmic ribonucleoproteins may be characterized by their lability to digestion by ribonuclease (cf. Dempsey and Wislocki, 1946, for references). Accordingly, parallel sections of Zenker-fixed tissue were stained by the eosin-methylene blue procedure with and without prior digestion in a solution of crystalline ribonuclease.[2] After digestion by the enzyme, both the follicular cytoplasm (figs. 5 and 6) and the colloid (figs. 1 to 4) failed to stain with methylene blue. On the other hand the staining of the chromatin granules in the nuclei of the

[2] The crystalline ribonuclease was kindly provided by Dr. M. Kunitz of the Rockefeller Institute for Medical Research, Princeton, N. J.

follicular cells, the mast cell granules, the matrix of the tracheal cartilage and the mucous droplets in the tracheal epithelium, were unaffected by treatment with the enzyme.

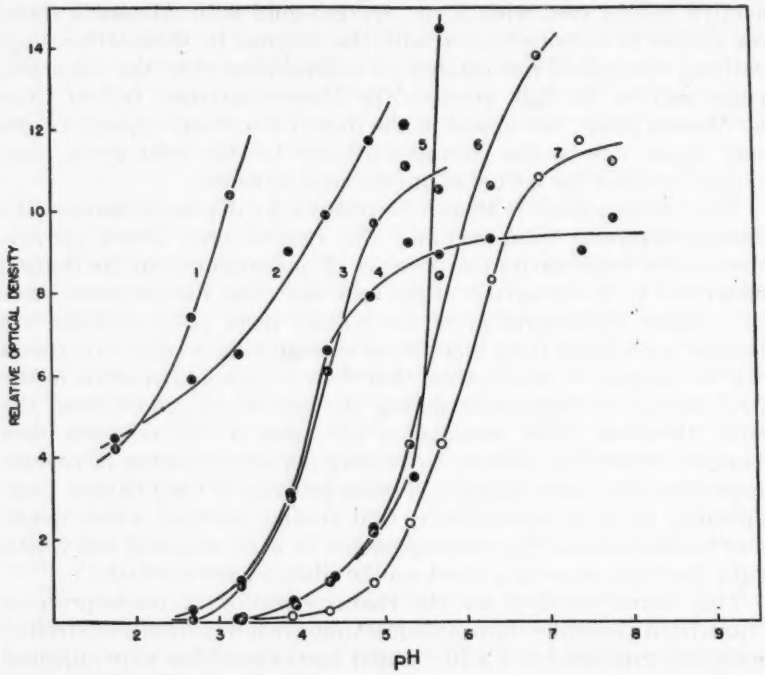
The profound alteration in staining characteristics which followed treatment of sections with ribonuclease was demonstrated not only with the eosin-methylene blue procedure but also with the triacid Azan and Masson's methods. The characteristic staining of the dense, inactive colloid (red with Azan, reddish-gold with Masson's stain) was altered in sections treated with the enzyme. In these latter preparations, the colloid was colored by aniline blue after the Azan procedure and by the light green in the Masson mixture. Indeed, after the Masson stain, the colloid of the digested sections appeared to be more dense, due to the increased affinity for the light green component, than did the colloid of untreated specimens.

Since ribonuclease is known to remove, by depolymerization, the ribonucleoproteins from sections, the results cited above provide presumptive evidence for the presence of nucleoprotein in the thyroid colloid and in the cytoplasm of the follicular cells. Furthermore, since the staining characteristics of the colloid from active follicles are identical with those from inactive or storage follicles after treatment with the enzyme, it would seem that there is a rapid depletion of the intrafollicular nucleoprotein during the release of colloid from the gland. However, these conclusions are open to the criticism that ribonuclease may in addition to causing depolymerization of ribonucleoprotein, also cause changes in other proteins of fixed tissues. Consequently, we have attempted to find another method which might serve to characterize the nucleoproteins in fixed material and which might therefore serve as a check on the ribonuclease method.

This second method for the characterization of nucleoproteins derives from a consideration of their staining reactions under controlled conditions. Solutions of 5×10^{-4} molar methylene blue were adjusted to different pH values with acetate or phosphate buffers having an ionic strength of 0.01. Sections of Zenker-fixed tissues were placed in 1500 cc. of these solutions and stained at 25 ± 0.1°C. for 24 hours during which time equilibrium was reached. The sections were then rapidly dehydrated and covered as permanent preparations. Microscopic examination showed that the intensity of staining varied directly with the pH. The tissues exhibited their greatest affinity for the dye at high pH values, whereas in the acid ranges little or no staining occurred. Such an influence of pH on staining has been reported in the past and has been discussed by Levene (1940).

The intensity of staining of the various tissue elements was determined by comparing the depth of color as observed through the microscope with that of a standard solution of methylene blue which was placed in the cup of a Duboscq colorimeter. In order to facilitate the determination of staining intensity, a comparison eyepiece was

attached to the oculars of the microscope and the colorimeter, thus producing a divided field in which the color of the section and of the standard solution could be viewed simultaneously. The vernier readings from the colorimeter were recorded and used as arbitrary measures of staining intensity. The graphs which resulted from plotting these indicates against their corresponding pH values are reproduced

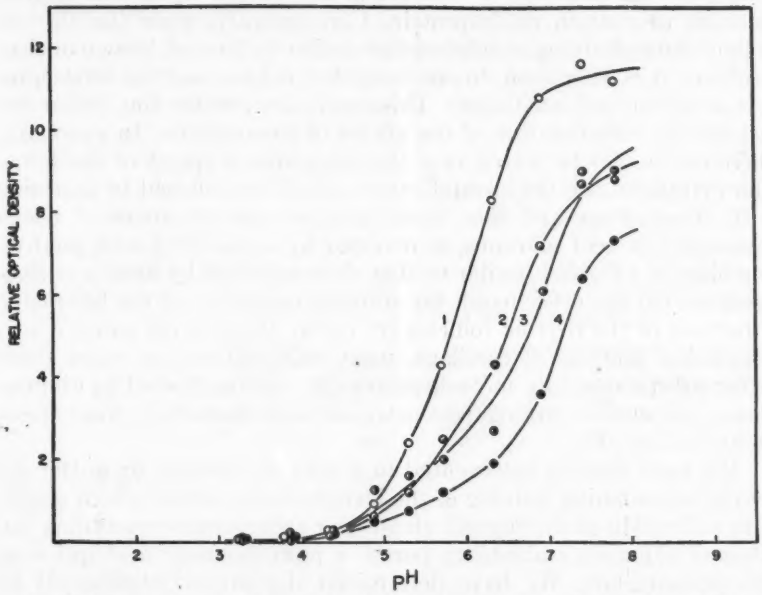


TEXT FIG. 9. A comparison of the binding capacity for methylene blue of various basophilic substances over a range of pH. (1) Capsular portion of tracheal cartilage surrounding lacunae. (2) granules of mast cells. (3) intracellular droplets of mucus from the tracheal epithelium. (4) intercapsular matrix of tracheal cartilage. (5) Nissl bodies from rabbit anterior horn cells. (6) eleidin granules from the esophageal epithelium. (7) colloid from peripheral inactive thyroid follicles. Except for (5), all regions studied were selected from blocks of tissue taken from the neck regions of rats.

as Figures 9 and 10. For comparison with the colloid, curves were also constructed for other well-known basophilic substances, such as the eleidin granules of the esophagus, mucus, Nissl bodies from spinal cord neurons, the granules of mast cells and the intercellular matrix of cartilage.

Figure 9 demonstrates that the graphs for thyroid colloid (7), eleidin granules of the esophageal epithelium (6) and Nissl substance of the anterior horn cells of the spinal cord of the rabbit similarly fixed

and stained (5) are similar in character. The three curves tend to approach zero at approximately the same pH. Likewise, all three curves show a notable upsweep at about the same pH range. At higher pH values there is a marked divergence of the intensity values for the three elements as the region of maximum uptake is approached. The similarity of the region of extinction and greatest change in stainability denotes a similarity in the underlying substances whose acid dissociation is responsible for the uptake of the basic dye. On the other hand, differences in the maximal intensities as observed at high



TEXT FIG. 10. A comparison of the binding capacity for methylene blue of thyroid colloid from various types of follicles. (1) colloid from peripheral inactive follicles of normal rat. (2) colloid from central active follicles of normal rat. (3) colloid from peripheral follicles of rat stimulated by exposure to cold. (4) colloid from central follicles of rat stimulated by exposure to cold.

pH readings bespeak differences in concentration of the stainable material.

The basophilic staining of cartilage, tracheal mucus and mast cell granules was quite different from that of substances mentioned above. All of these continued to stain at values below the extinction point of Nissl, eleidin and colloid basophilia. The mast cell granules (2) and the capsular portion of the cartilage matrix (1) were stained appreciably in solutions as acid as pH 1.8, indicating that the acid charges of these substances were extremely strong since acid dissociation was not suppressed even at these low pH values. The remainder of the

cartilage matrix (4) and the mucous droplets (3) of the tracheal epithelium were characterized by extinction values and regions of increase of stainability which were intermediate between those of the other two groups of elements.

These results, obtained by measuring the binding powers of tissue elements for dyes at different acidities, indicate that materials in several different localities have similar properties. One such group, consisting in our experiments of colloid, eleidin granules and Nissl substance, has its stainability reduced to zero at pH 4.0 and below. The last two members of this group are known by spectrographic analysis to contain nucleoprotein. Consequently, since the thyroid colloid shows staining characteristics similar to those of known nucleoproteins, it is reasonable to conclude that it also contains nucleoprotein as a principal constituent. This conclusion fortifies the similar one reached by consideration of the effects of ribonuclease. In summary, therefore, it may be stated that the basophilic property of the follicular cytoplasm and the intrafollicular colloid is abolished by digestion with ribonuclease and that these localities contain material which dissociates in acid solutions, as revealed by stainability with methylene blue, in a fashion similar to that demonstrated by known nucleoproteins. On the other hand, the staining properties of the basophilic substance of the thyroid follicles are unlike those of the sulfuric acid containing proteins of cartilage, mast cells and mucus, since these latter substances stain metachromatically, are unaffected by ribonuclease and exhibit stronger dissociations as indicated by their stainability at low pH.

We have already commented in a previous section upon the decrease in basophilic staining of the intrafollicular colloid which occurs with activation of the thyroid gland. Our experiments concerning the effect of pH upon stainability permit a more accurate description of this phenomenon. We have determined the curves relating pH to staining intensity for the most intensely staining colloid from peripheral follicles and for the most weakly staining colloid from central follicles in both normal rats and rats which had been maintained in the cold room (fig. 10). These results fall into a family of curves. The fact that the extinction points and regions of greatest change of

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PLATE 3

Fig. 11. Reticulum in the cat's thyroid gland. After formalin fixation, only the argyrophilic fibers are impregnated. Formalin fixation, Pap's stain. ×350.

Fig. 12. Argyrophilia of the intrafollicular colloid in the cat's thryoid gland. Alcohol, formol, picric acid fixation. Pap's stain. ×350.

Fig. 13. Thyroid gland from a normal control rat, illustrating the reaction of the colloid with Bauer's method. Bouin fixation. Bauer stain. ×200.

Fig. 14. Thyroid gland from a rat exposed to cold, illustrating the marked reduction in the material which reacts with Bauer's method. Bouin fixation. Bauer stain. ×200.

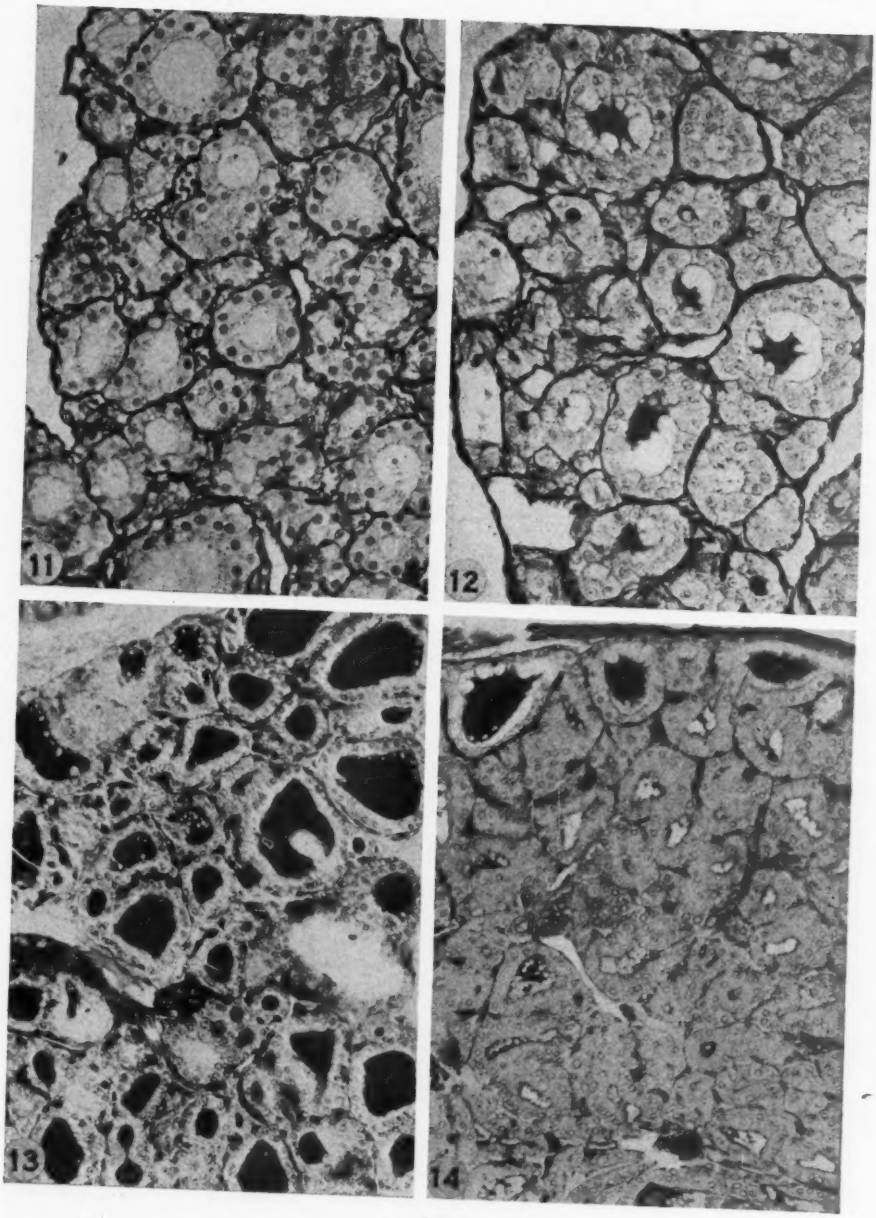

PLATE 3

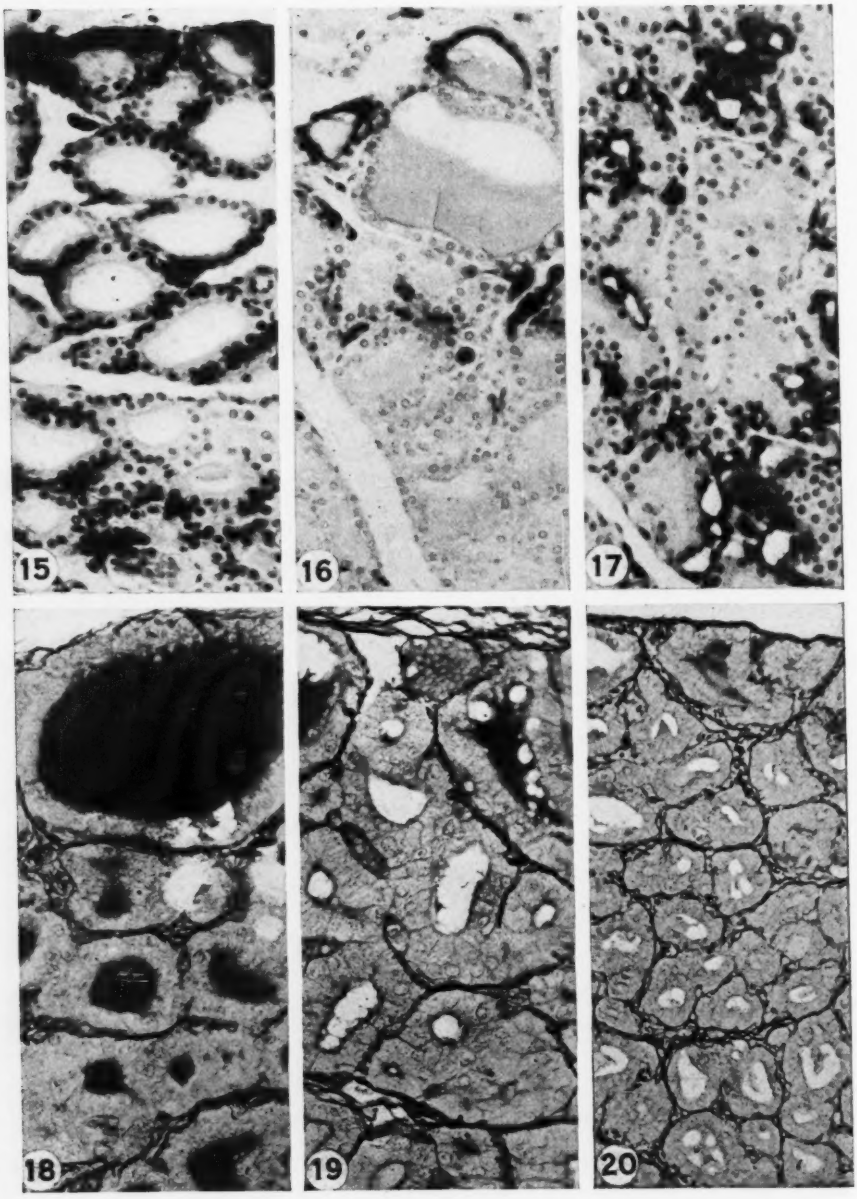

PLATE 4

stainability are similar supports the contention that the stained protein is the same in the different follicles. The differences in maximal staining obtained at high pH denote quantitative differences in concentration of the basophilic substance. The most concentrated material was in the peripheral inactive follicles from normal rats (1) and the least concentrated in the central follicles of rats which had previously been maintained in the cold room (4). Exposure to cold reduced the basophilia of the colloid from both central and peripheral follicles (compare curves 2 and 4; 1 and 3). Indeed, stimulation by cold caused so great a loss in the basophilia of the least active follicles that their content approximated that of the most active follicles from normal rats (compare 2 and 3).

3. *Argyrophilic substances in the thyroid gland.* In the course of the present study argyrophilic reactions were observed in a number of locations in the thyroid gland. In an effort to display the reticulum of the gland, Pap's procedure was applied with excellent results (figs. 11 and 12). In addition to revealing the delicate argyrophilic network of fibers in the stroma, silver was also deposited in several other regions, the localization of which varied according to the fixative used. After fixation in formalin, the reticulum alone was silvered, the follicular cells and colloid remaining unimpregnated. When a variety of other fixatives were used, for example, alcohol-formalin, alcohol-picro-formalin and Bouin's fluid, the intrafollicular colloid became blackened with silver. The intensity of the reaction varied in different parts of the colloid, being most vigorous in the colloid from the peripheral follicles and weakest in that from the central part of the gland (fig. 18). A further correlation between activity of the follicle and intensity of staining was forthcoming from a study of the glands from animals which had been exposed to cold and which had

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PLATE 4

Fig. 15. Fructose diphosphatase reaction, pH 9.5, in the endothelial and follicular cells of the thyroid gland from a normal control rat. The section was incubated for 24 hours in the substrate mixture, and the precipitated phosphate was visualized by transformation to cobaltous sulfide. Fixation in cold 80% alcohol. ×200.

Fig. 16. Thyroid gland from a rat exposed to cold, illustrating the reduction in the alkaline fructose diphosphatase reaction. The enzyme does not appear in the endothelium or parenchymal cells associated with the central or most active follicles, but is restricted to the peripheral, inactive portions of the gland. Fixation in cold 80% alcohol. ×200.

Fig. 17. Alkaline fructose diphosphatase in the thyroid gland of a rat to which thiouracil had been administered. Fixation in cold 80% alcohol. ×200.

Fig. 18. Argyrophilia of the colloid from the thyroid gland of a normal control rat. Bouin fixation, section digested in saliva, Pap's stain. ×350.

Fig. 19. Section illustrating the reduction in argyrophilia, particularly in the central follicles, from a rat exposed to cold. Bouin fixation, section digested in saliva, Pap's stain. ×350.

Fig. 20. Section illustrating the further reduction in argyrophilia in the thyroid gland of a rat to which thiouracil had been administered. Bouin fixation. Sections digested in saliva, Pap's stain. ×200.

been treated with thiouracil. The degree of impregnation was greatly depressed in the rats subjected to these latter procedures (figs. 18, 19 and 20).

Argyrophilic granules in the follicular epithelium were also exhibited after Pap's reaction. These granules were impregnated routinely after Zenker-acetic fixation (figs. 23 and 24). The granules were impregnated throughout all parts of the follicular cells but were usually somewhat concentrated under the apical membrane. The size and apparent density of the granules did not vary markedly in different states of activity. However, since the cells from active follicles are much taller than those from inactive ones, it would seem likely that an actual increase in the number of granules occurs on activation of the gland.

The Bodian silver protargol procedure, when applied to the thyroid gland, also impregnated granules within the cytoplasm of the follicular epithelium (fig. 8). The size and distribution of these Bodian-positive granules were similar to those revealed by Pap's method. Except for the argyrophilic granules, however, the two methods yielded dissimilar results. The reticulum and the colloid, both of which were impregnated by Pap's method, were unaffected by Bodian's stain. The nuclei in the thyroid gland were strongly impregnated by the Bodian stain. After Pap's procedure, the nuclei were stained only faintly after the alcoholic fixatives and not at all in Zenker-fixed sections.

The argyrophilia of the thyroid colloid bears some resemblance to that of glycogen and mucus, since it is revealed after alcoholic fixation by Pap's procedure. A further similarity derives from the fact that the colloid also exhibits positive reactions with Bauer's procedure (vide infra). Nevertheless, we will attempt to show in a subsequent section that neither mucus nor glycogen can account for the argyrophilia of the colloid. The presence of Bodian-positive granules in the thyroid epithelium also suggests the presence of calcium deposits, since formed calcium is among the substances blackened by the method (Wislocki and Dempsey, 1945). These particular argyrophilic granules of the thyroid gland are apparently not formed deposits of calcium, however, since they persist in sections from tissues fixed in Bouin's fluid (an aqueous acid) and since they are not revealed by other methods which stain calcium such as the ammonium sulfide-cobaltous chloride procedure.

4. *The Bauer reaction.* The discovery that thyroid colloid, especially after alcoholic fixation, was impregnated by the Pap's procedure suggested that glycogen might be contained within it, since Mitchell and Wislocki (1945) found that glycogen could be selectively demonstrated by this method. Consequently other tests were performed to determine the presence or absence of glycogen. The Bauer-Feulgen procedure, consisting in the recolorization of leuco-fuchsin

after hydrolysis by chromic acid (cf. Cowdry, 1943), was applied to sections of tissue fixed in various ways. Selective staining of the colloid occurred after fixation in alcoholic mixtures and in Bouin's or in Zenker's fluids (fig. 13). Digestion of sections in saliva for periods of up to three hours failed to alter the Bauer reaction of the colloid.

The persistence of the Bauer reaction after digestion in saliva and after fixation in aqueous fixatives precludes the possibility that glycogen might be the reactive substance. This conclusion has also been reached by Wollraff and Beckert (1939) after similar experiments. A second possibility, that mucoid substances might occur in the thyroid colloid, can also be ruled out. Mucus reacts positively with both Bauer's and Pap's procedures, but mucus stains metachromatically with toluidin blue whereas, as described in a previous section, the colloid does not. Furthermore, the relation between pH and staining affinity is quite different for colloid and mucus. It would appear from these considerations that the argyrophilia as demonstrated with ammoniacal silver nitrate and by the regeneration of fuchsin in the Bauer reaction must indicate the presence in the colloid of some unknown substance, which is preserved by protein precipitants and which probably contains active carbonyl groupings. Such an hypothetical compound is of unknown metabolic significance, and it is therefore of some interest to determine whether different states of physiological activity modify the results obtained through the use of the Bauer procedure.

The colloid which was most intensely stained by the Bauer procedure was found in the peripheral thyroid follicles of rats maintained either in the hot room or at normal room temperature. The colloid from centrally located follicles of the control animals stained more faintly than that of the inactive peripheral follicles. Exposure to cold environments caused a reduction in the intensity of staining, so that as a rule only the outermost follicles contained reactive material (fig 14). Similarly, treatment of rats with thiouracil caused a marked decline in the stainable colloid. These results indicate a rapid and profound decrease in material stainable by the Bauer procedure during release of colloid, and are entirely similar to the changes described above for the argyrophilia of the colloid following the application of Pap's method.

5. *Phosphatase reactions.* In their excellent surveys of the principal sites at which alkaline glycerophosphatase exists in the mammalian body, Gomori (1941a) and Kabat and Furth (1941) remark that the enzyme is present only in the capillaries of the thyroid gland. Gomori detected a spurious reaction in the colloid and determined that it was not caused by phosphatase since the blackening of the colloid occurred in control sections which had not been incubated in the substrate. Wolf, Kabat and Newman (1943) mention the presence of acid phosphatase in the nuclei and cytoplasm of the thyroid epi-

thelium, but give no details as to its localization. Notwithstanding these meager reports of the presence of phosphatase in the thyroid gland, we have found that both acid and alkaline glycerophosphatase may be revealed by longer incubation periods than those used formerly. In addition, using as substrates phosphoric esters other than glycerophosphate, we have demonstrated the presence of enzymes capable of dephosphorylating yeast nucleic acid, adenylic acid, fructose diphosphate, glucose-1-phosphate and lecithin.[3]

Using Gomori's (1941a) alkaline phosphatase method, in which glycerophosphate buffered to pH 9.4 is employed as a substrate, we have found deposits of phosphatase in the epithelium of the peripheral inactive follicles (fig. 20). This reaction was not elicited in our experiments after 1½ and 3 hours' incubation, but occurred regularly in the thyroid glands of animals maintained at room temperature or in the hot room following 6 hours or longer incubation in the substrate mixture. The glands from animals maintained in the cold room were consistently negative in our experiments. As illustrated in figure 20, the reaction was most intense at the apical border of the follicular epithelium and extended into the depths of the cells where it became progressively fainter toward the base. Frequently only the peripheral portion of the outermost follicles displayed the reaction. Alkaline glycerophosphatase does not occur in the capillary endothelium of the rat, but is present in the dog's thyroid gland. Except for these cellular locations and for the faint blackening of the colloid by the spurious reaction described by Gomori, the remainder of the thyroid elements were negative.

Acid phosphatase was also demonstrated in the thyroid gland by experiments employing incubation in the glycerophosphate substrate for 48 hours or longer. The distribution of the enzyme revealed by incubation at pH 4.7 was entirely different from that at the alkaline pH. Acid glycerophosphatase was most strongly present in the nuclei of the centrally located follicles. In addition to the nuclear staining, the epithelial cytoplasm of the central active follicles exhibited a delicate granular reaction, concentrated for the most part in the apices of the cells but extending downward as more sparsely distributed granules in the basal and perinuclear regions (fig. 21). The nuclei and cytoplasm of the peripheral follicles in the glands of animals maintained at room temperature were entirely negative, as were also the stromal and endothelial cells. The intensity of the reaction was greater and extended nearer to the periphery of the glands in the cold room animals, and conversely, was decreased in the thyroids of animals subjected to hot environments.

[3] A sample of glucose-1-phosphate was placed at our disposal by Professor David M. Greenberg of the University of California Medical School, to whom we wish to express our appreciation. The yeast nucleic acid, adenylic acid and fructose diphosphate were obtained from the Schwarz Laboratories, 202 East 44th St., New York City. Vegetable lecithin was obtained from the Pfanstiehl Chemical Co., Waukegan, Ill.

In addition to the glycerophosphatase activity described above we have also tested the ability of the thyroid gland to free phosphate from lecithin, fructose diphosphate, glucose-1-phosphate, yeast nucleic acid and adenylic acid. Lecithin proved to be entirely negative in our experiments when incubated as long as 72 hours at pH 5.0 and 9.0. At pH 7.0 a weak reaction occurred in the nuclei and traces of the enzyme were detected in cytoplasm. These reactions were equally present in the central and peripheral follicles. Similarly, positive results were secured with the hexose phosphates and with the nucleotide and nucleic acid substrates, and the localities exhibiting these activities were different from those visualized after incubation with glycerophosphate.

The most striking difference exhibited by the hexose phosphatases was the strong predilection of the capillary endothelium for the enzymes. When incubated in fructose diphosphate at pH 9.0 for five hours or longer, the endothelial cells surrounding the peripheral follicles were densely stained. In addition, the epithelial cells of the peripheral follicles contained fructose diphosphatase (fig. 15) in a fashion similar to that already described for glycerophosphatase (fig. 20). The nuclei of the follicular epithelium were generally unstained, except for the peripheral cells exhibiting the most intense cytoplasmic reaction in which locality the nuclei also appeared black.

The reactions obtained by incubating sections in glucose-1-phosphate for similar times and at the same pH were slightly different from those described for fructose diphosphate. The principal differences consisted in the prominent reactions of the nuclei and the less intense blackening of the endothelium. The epithelium of the peripheral follicles contained glucose-1-phosphatase in the same distribution as that described for glycerophosphatase and fructose diphosphatase.

Exposure of rats to cold and administration of thiouracil induced changes in the amount and distribution of the hexose phosphatases. However, the number of animals subjected to these treatments was small (two animals for each experimental procedure) and the results were somewhat contradictory rather than mutually confirmatory as has been the case for the other procedures used in this investigation. Consequently, the results will be described but we shall refrain from attaching too much significance to them until they may be confirmed in a larger series of animals.

In the thyroid glands activated by the exposure of rats to cold environments, there was marked decrease in the amount of both fructose diphosphatase and glucose-1-phosphatase. The endothelial reaction with both substrates was restricted to the peripheral portions of the glands (fig. 16). The phosphatases of the peripheral epithelium were also reduced in amount until only occasional follicles, located at the extreme margin of the thyroid gland, contained detectable amounts of the enzymes (cf. figs. 15 and 16). After treatment with

thiouracil, on the contrary, there was a moderate increase in the amount of the phosphatases of the endothelium. Fig. 17 illustrates the dense concentration of fructose diphosphatase which surrounds the widely dilated capillaries after stimulation of the gland by thiouracil.

The distribution of fructose diphosphatase and glucose-1-phosphatase was similar at neutral pH to that described for pH 9 with the exception that the nuclear reaction was more prominent. At pH 5, the thyroid gland was entirely unreactive except for faint traces of the enzymes in the nuclei of the parenchymatous cells. These appearances were quite different from those obtained for glycerophosphatase since the latter enzyme was readily detected in the apical portions of the active follicular cells.

Yeast nucleic acid also proved to be a suitable substrate for thyroidal phosphatases. The phosphatase revealed at pH 9 by this substrate was concentrated in the nuclei and endothelium and weaker in the cytoplasm. The intensity of staining was perhaps slightly greater in the peripheral epithelium than in that more centrally located, but this difference was so slight as to be almost undetectable. Exposure to cold and administration of thiouracil did not influence the distribution of the enzyme in the parenchymatous cells, but depressed that occurring in the capillary endothelium.

The nuclear reaction for nucleic acid phosphatase was enhanced in preparations incubated at pH 7, and, although less intense, persisted at pH 5. The endothelial reaction was not detected at neutral and acid pH. Only traces were demonstrable in the cytoplasm of the follicular epithelum after incubation in neutral and acid solution.

Adenylic acid, with a single exception, yielded results at all pH's similar to those obtained with nucleic acid. The exception consisted in the concentration of alkaline enzyme detected in the epithelium of the peripheral follicles. The results with adenylic acid, therefore, are intermediate between those obtained with hexose phosphates on the one hand and with nucleic acid on the other.

PLATE 5

Fig. 21. Drawing illustrating the localization of alkaline glycerophosphatase (pH 9.4) in the follicular cells of peripheral follicles from the thyroid gland of a normal control rat. The endothelial cells are negative (compare with figure 14). Fixation in cold 80% alcohol. Section incubated in the substrate mixture for 6 hours. 40× objective, 7× ocular.

Fig. 22. Drawing illustrating the localization of acid glycerophosphatase in the nuclei and parenchynal cells of the central follicles from the thyroid gland of a rat exposed to cold. Fixation in cold 80% alcohol. Section incubated 48 hours. 40× objective, 7× ocular.

Fig. 23. Drawing illustrating the argyrophilic granules of the follicular epithelium of a normal control rat. This appearance has been observed only after Zenker fixation. Compare with figure 8. Pap's stain. 90× objective, 10× ocular.

Fig. 24. Argyrophilic granules in the thyroid gland from a rat after exposure to cold. Zenker fixation. Pap's stain. 90× objective, 10× ocular.

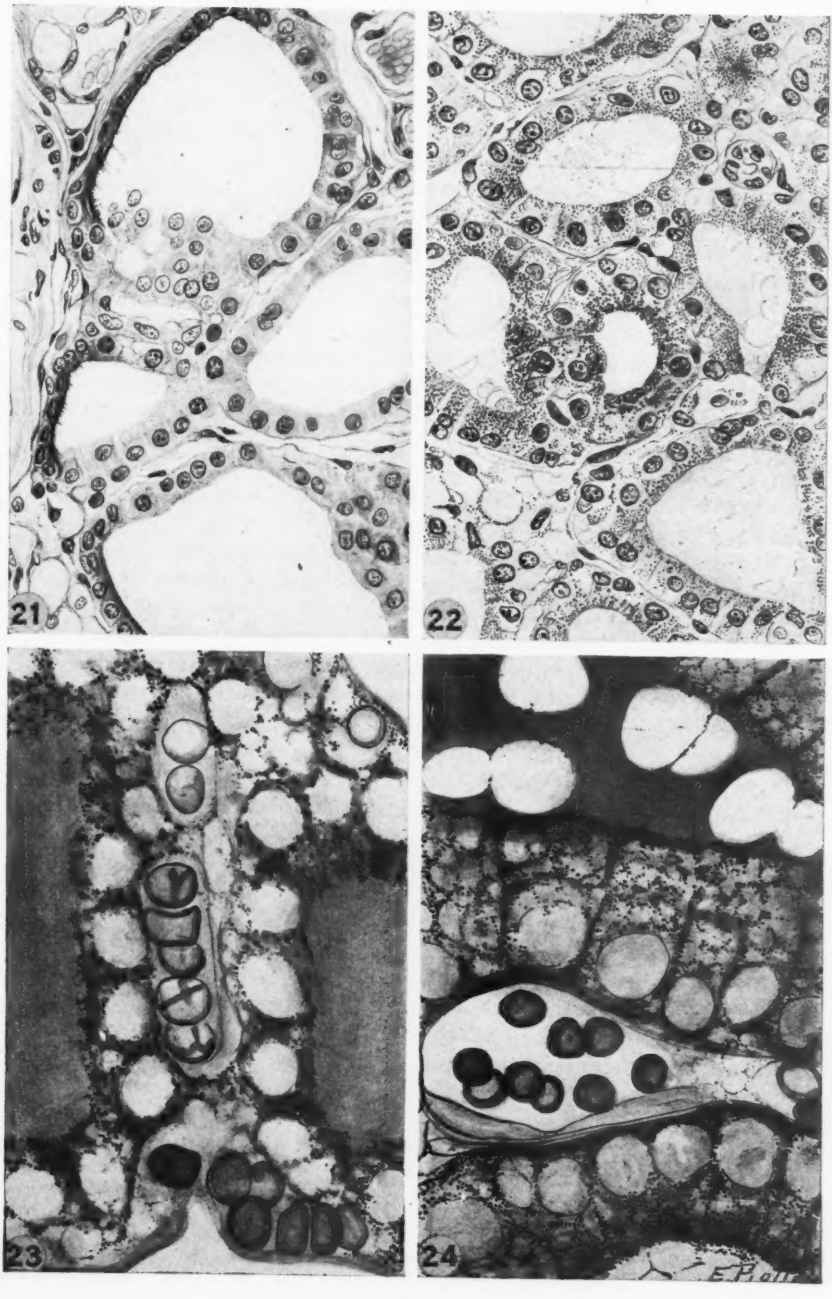

PLATE 5

DISCUSSION

The basophilic material in the thyroid gland. This investigation provides data which strongly suggest that the basophilic reactions of the colloid and cytoplasm are attributable to their content of ribonucleoprotein. This evidence derives from the staining reactions which, under precisely controlled conditions, are strikingly similar to those of other regions known to contain nucleoprotein (e. g., Nissl substance of neurons). Since the dissociation of the acid groups, as tested by combination with the basic dye, is equivalent to those of nucleoprotein, it seems probable that the charge of the nucleic acid molecule is responsible for the basophilic staining of the colloid and cytoplasm. If this conclusion be valid, it follows that the nucleoprotein must be of the ribose variety, since it does not stain with the Feulgen procedure for desoxyribonucleoproteins. Furthermore, the destruction of the basophilic staining reaction by digestion in ribonuclease harmonizes with and fortifies this hypothesis. Indeed, the agreement between the results obtained by treatment with ribonuclease and those derived from the staining reactions constitute such strong evidence for the hypothesis that nucleoprotein is a constituent of thyroid colloid, that, had there been no contrary opinion, we should have had little hesitation in advancing it as a conclusion. However, Gersh and Caspersson (1940) and Gersh and Baker (1943) have studied the ultraviolet absorption spectrum of thyroid colloid and have found no evidence of the presence of nucleoprotein. Since the absorption of radiation at about 2600 Å is perhaps the most specific of the reactions of nucleoproteins, their failure to detect absorption at this wavelength constitutes such an important objection that it becomes necessary to review critically the evidence.

The chemical information on the composition of the thyroid colloid is not conclusive. Oswald (1899) found phosphorus in his active extracts of the gland, but Cavett, Rice and McClendon (1935) consider this to be derived from the cells since the proportion of phosphorus is diminished by methods of collection which minimize damage to the tissues. Nevertheless, even though the proportion of phosphorus is smaller under these conditions, it has never been removed completely even from purified preparations of thyroglobulin (Salter, 1940). It appears, therefore, that the presently available chemical evidence is not sufficient to decide whether or not nucleoprotein is a normal constituent of the colloid.

The evidence against the presence of nucleoprotein derived from the ultraviolet absorption studies of Gersh and Caspersson (1940) and Gersh and Baker (1943) also appears to be inconclusive. In their original study, Gersh and Caspersson studied the thyroid gland of guinea pigs, an organ like that of the rat in that the central follicles appear morphologically to be more active than those in the periphery. The colloid from the central follicles was selected by them for study

and was found not to absorb ultraviolet light selectively at 2600 Å. We have demonstrated in this investigation that the centrally located colloid of the rat stains only weakly with methylene blue. Furthermore comparison of figures A and C in Gersh and Caspersson's article reveals that although there is no peak in the curves at 2600 Å, nevertheless the extinction coefficient at this wavelength is twice as great for normal colloid as for the colloid from an animal which had been strongly stimulated with an extract of the anterior pituitary gland. Consequently, it would seem that the small amount of absorption observed by Gersh and Caspersson could be accounted for by the small amount of nucleoprotein, as judged by our staining reactions, which is present in the active follicles in the central regions of the thyroid gland. Likewise, activation of the thyroid gland by anterior pituitary extract caused a reduction in absorption at 2600 Å in their experiments, whereas in ours exposure to cold environments caused a reduction of staining intensity.

In a second investigation, Gersh and Baker (1943) repeated and extended the observations described above using the rat as their experimental animal. The colloid from follicles selected at random was investigated by determining the extinction coefficient for three points, namely 2800 Å, 3341 Å and 3654 Å. Consequently, although presumably some of the follicles investigated contained dense basophilic colloid, the absorption at 2600 Å which serves as an index of nucleoprotein concentration was not determined. Complete absorption curves were made in this investigation only on colloid withdrawn in a micropipette. The location of the follicles aspirated was not stated; consequently one cannot evaluate these results in terms of the probable basophilic properties of the colloid obtained.

From the above considerations, it would seem that the information derived from ultraviolet absorption studies is not sufficient for concluding the absence of nucleoprotein in the follicular colloid. There remains, however, the possibility that the basophilia of the thyroid colloid might be accounted for by some acidic protein other than nucleoprotein. We shall attempt to show, in the following paragraph, that other commonly occurring acid substances do not occur in appreciable quantities in the thyroid gland.

The data derived from staining with buffered solutions of dye indicate that the material within the colloid and follicular cytoplasm is quite strongly acid, since the ionic dissociation is not suppressed above pH 4.0. Among the acid groups capable of such strong dissociation, sulfate, phosphate and carboxyl radicles immediately come to mind. It seems unlikely that carboxyl groups are the responsible agents since fixatives employing mercuric salts combine with and destroy the charge of carboxyl radicles (Tolstoouhov, 1928), yet these same fixatives do not suppress the basophilia of the thyroid. The sulfate radicle also seems an unlikely possibility for several

reasons. Chemical data indicate the ability of the sulfate ion to dissociate at values far below pH 4.0, a fact which is in accord with our findings that sulfate esters of proteins such as those present in mucus, cartilage and mast cells continue to stain in solutions as acid as pH 3.0. Furthermore, sulfuric esters of high molecular weight exhibit metachromasia when stained with thiazin dyes such as toluidin blue. We have demonstrated in an earlier section that the thyroid colloid and cells do not stain metachromatically. Consequently, we derive from these considerations that sulfate and carboxyl groups are probably not responsible for the basophilia of the thyroid. By exclusion, phosphate radicles become the most likely possibility.

In summary, therefore, we may state that critical evidence is lacking for the absence of nucleoprotein in the thyroid colloid and cytoplasm of the parenchymatous cells. On the other hand, evidence for the presence of ribonucleoprotein may be deduced from the abolition of basophilic staining by ribonuclease and from the determination of the pH at which the acid dissociation of the colloid and follicular cytoplasm is suppressed. This value is similar to that which suppresses staining of Nissl substance and of other regions known to contain nucleoproteins, and is presumably due to the charge of the phosphate radicle of nucleic acid.

The possible significance of phosphatase in the thyroid gland. Following Gomori's (1941) demonstration of phosphatase in tissue sections, a number of papers has appeared describing the localization of acid as well as alkaline phosphatases in the various organs and tissues of the mammalian body. Except for the relation of alkaline phosphatase to calcification, little success has attended efforts to gain insight into the metabolic significance of the enzymes. Recently, however, several investigations centering around the correlation of phosphatase concentrations with different physiological activities have resulted in empirical data which demonstrate a relation, the details of which remain vague, between phosphatase activity and nucleoprotein concentration. Dempsey and Wislocki (1945) found that basophilia and alkaline phosphatase are inversely related in the placental syncytium; as basophilia declines with advancing age of gestation, alkaline phosphatase increases. Acid phosphatase also increases with increasing placental age (Wislocki and Dempsey, 1946). A somewhat similar relation exists in neurons during chromatolysis. Associated with the dissolution of the Nissl substance there is an increase in acid phosphatase (Bodian and Mellors, 1945). Our observations, as described in the present article, indicate that in the thyroid gland also, basophilia and phosphatase undergo coupled changes. Upon activation of a thyroid follicle, the cells increase in volume and the cytoplasm stains somewhat less intensely with methylene blue; concomitantly acid phosphatase puts in its appearance and alkaline phosphatase decreases or disappears. Parenthetically, it may be remarked that the coupled

changes of phosphatase and basophilia in the thyroid gland are in harmony with the thought that the staining reactions are caused by nucleoprotein, since somewhat similar relations exist in the nerve cell where the nucleoprotein composition of Nissl substance is well founded (Gersh and Bodian, 1943).

Somewhat more specific delimitation of the possible significance of the thyroid phosphatases may be achieved by considering the substrates which permit determination of the enzymes. Since little activity could be detected with lecithin, it seems reasonable that this substance is not metabolically important in the thyroid gland. Likewise, the localization detected by using nucleic acid as a substrate was such as to suggest merely the participation of nucleic acid phosphatase in the metabolic turnover of nucleoprotein, since large shifts in the amount of neither enzyme nor cytoplasmic basophilic substance occur with activity. On the other hand, glucose-1-phosphatase, fructose diphosphatase and glycerophosphatase were strikingly different in their distributions and concentrations in the various states of physiological activity considered here. It seems likely, from these considerations, that the dominant role of phosphatase is its participation in the intermediary carbohydrate metabolism of the thyroid gland.

The last consideration pertaining to phosphatase which we wish to discuss is in relation to the physiological activity of the endothelium. The inadequate visualization of endothelial cells by ordinary histological procedures has resulted in failure to consider the possible importance of the endothelial system in regulating the specific activities of tissues and organs. In other words, since by ordinary methods all endothelial cells look alike there has been a tendency to assume that diffusion or transmission through these cells is equal in all regions. The demonstration of concentrated deposits of an enzyme in some but not other endothelial cells throws new light on this system. It seems clear that regions containing the enzyme may transmit across the endothelial barrier materials which are denied passage by other regions lacking the enzymatic mechanism. More specifically, the demonstration that pronounced and rapid alterations in the endothelial glucose-1-phosphatase and fructose diphosphatase occur on altering the physiological status of the gland leads to the thought that these changes in endothelial phosphatase may provide a qualitatively different pabulum to the parenchymatous cells of the active as opposed to the inactive gland. It is even possible for the future to reveal that the trophic hormones act, not directly on the parenchyma of target organs, but indirectly through controlling the metabolites accessible to the organ by altering the physiological activity of the endothelium.

The composition of the intrafollicular colloid. The present experiments throw some light on the problem of whether the thyroid colloid may be regarded as a storage form of thyroglobulin. It has long been known that colloid accumulates in states of reduced thyroidal activ-

ity and is reduced in amount during states of hyperactivity. The further demonstration that iodine is a constituent of the colloid (cf. Salter, 1940) has led to the rather general belief that thyroglobulin and colloid are synonymous. Although there seems to be good evidence that the thyroid hormone does indeed constitute an important component of the colloid, there is now equally good evidence that other substances occur within the intrafollicular space and that colloid may be manufactured which is totally deficient in thyroid hormone.

Astwood, Sullivan, Bissell and Tyslowitz (1943) demonstrated that complete remission of the hyperplastic effects of anti-thyroid drugs was secured by hypophysectomy or by the administration of thyroxin. Subsequently, Astwood and Bissell (1944) showed that the new colloid formed after hypophysectomy is deficient in iodine. These observations amply demonstrate that colloid may or may not contain thyroglobulin. It would appear, therefore, that the bulk of the colloid must be composed of other substances.

In discussing the chemical nature of the colloid, Dempsey (1944) suggested that two general alternatives seemed evident. Colloid might consist of a protein which is elaborated by the thyroid gland and which is capable of combining under some conditions with an active prosthetic group containing iodine, or colloid might normally be a mixture of inert proteins and an active, iodine containing substance. The first hypothesis envisions the elaboration of colloid as a chain reaction, one step of which may be omitted with a consequent deficiency in the final product, whereas by the second hypothesis independent processes, normally occurring at consonant rates, are postulated.

The second of these hypotheses now appears to be the more attractive. The present experiments demonstrate that the colloid contains a basophilic substance which behaves like a polynucleotide. We have evidence, also, for an argyrophilic protein the reactions of which are characteristic of active carbonyl groupings. De Robertis (1941) has shown the presence of a proteolytic enzyme in the thyroid colloid. There now seems to be no doubt but that the thyroxin is the active grouping of the thyroid hormone. Although there is no direct evidence on the point, it would seem likely that these separate activities represent separate substances. In the unlikely event that only one protein were involved, it would be a hydra-headed protein molecule indeed which had so many and so diverse prosthetic parts.

SUMMARY

The cytoplasm of the follicular cells and the intrafollicular colloid of the rat's thyroid gland exhibit basophilia after staining with eosin-methylene blue. This basophilia is interpreted as indicative of ribonucleoprotein, since it is abolished by treatment of sections with crystalline ribonuclease and since its stainability in buffered solutions

of dye is similar to that of other regions known to contain ribonucleo proteins.

Thyroid follicles of the rat do not contain any substance which gives reactions characteristic for sulfuric acid-protein esters. The thyroid epithelium and colloid do not stain metachromatically with toluidin blue, nor does the pH at which suppression of basic staining occurs coincide with that for cartilage, mucus or mast cells.

Argyrophilic granules may be demonstrated in the thyroid epithelium of various animals by Bodian's and Pap's methods, after appropriate fixation. Argyrophilic staining of the colloid, coupled with positive reactions with Bauer's method, indicate the presence of a substance containing active carbonyl groups.

Alkaline glycerophosphatase reactions occur in the follicular epithelium of peripheral, inactive follicles of the rat thyroid. Acid glycerophosphatase occurs in the cells of central, active follicles. Glucose-1-phosphatase and fructose diphosphatase are concentrated in the capillary endothelium. These enzymes are altered in their distriubtion during altered activity of the gland. Nucleic acid, adenylic acid and lecithin may also be used as substrates to demonstrate phosphatase activity, but the phosphatases demonstrated with the aid of these substances are relatively unaffected by alterations in the physiological activity of the thyroid gland.

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THE ROLE OF THE ADRENALS IN THE PRODUCTION OF NEPHROSCLEROSIS BY ANTERIOR PITUITARY PREPARATIONS

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IT HAS been shown that the synthetic adrenal compound desoxycorticosterone acetate (DCA) produces pronounced renal and cardiovascular lesions in a variety of animal species (Selye and Hall, 1943; Selye, Hall and Rowley, 1943; Selye and Hall, 1944; Selye and Pentz, 1943). Sensitivity to such treatment is increased if the animals are unilaterally nephrectomized and kept on a high sodium chloride intake. The lesions consist of nephrosclerosis, periarteritis nodosa and nodules in the heart resembling Aschoff bodies. The development of these lesions is inhibited by acidifying salts such as ammonium chloride (Selye, Hall and Rowley, 1945).

Later it was found that unilaterally nephrectomized, sodium chloride treated rats develop these same lesions when exposed to various types of non-specific damage—especially cold—which induce adrenal cortical enlargement. This was interpreted as being due to the liberation of desoxycorticosterone-like compounds from the adrenal cortices through stimulation by endogenous hypophyseal corticotropic hormone. This view is also supported by the observation that ammonium chloride inhibits the production of these lesions just as it does those induced by DCA treatment (Selye, 1943, 1946).

To test this hypothesis rats were treated with a crude extract of cattle anterior pituitary glands and it was found that this substance likewise causes nephrosclerosis and allied lesions in sensitized animals (Selye, 1944). The lesions so produced were also prevented by ammonium chloride (Hall and Selye, 1945).

These experiments were repeated on adrenalectomized rats (maintained on a constant daily dose of adrenal cortical extract) in order to establish definitely the role of the adrenals in the pathogenesis of these lesions.

EXPERIMENTAL

One hundred female albino rats, weighing between 140 and 160 gms., were unilaterally nephrectomized and divided into eight groups, each having an average body weight of 153 gms. They were main-

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tained on a diet of "Purina Fox-chow" and given 1% NaCl solution to drink in place of tap water. Groups 3 and 8 each consisted of 20 animals the remaining groups of 10 animals. Group 1 received no treatment and served as controls. Group 2 received 0.33 cc. of cortical extract (Connaught Laboratories, Toronto) subcutaneously three times daily. Group 3 received twice daily, subcutaneously, 0.4 cc. of an aqueous suspension containing 40 mgs. of lyophylized (cattle) anterior pituitary (L.A.P.) powder. Group 4 received both cortical extract and L.A.P. in the same amount and manner as the animals of groups 2 and 3. Groups 5 to 8 were treated exactly as groups 1 to 4 respectively except that the animals were bilaterally adrenalectomized the day before the injections were begun. The experiment was terminated on the 29th day, when all surviving animals were killed and their organs taken for histologic examination. The results are summarized in Table 1.

At autopsy nephrosclerosis and cardiac lesions were observed in almost all animals of groups 3 and 4, while in the other groups no lesions were seen, although the kidneys and hearts were somewhat enlarged in group 8. The adrenal glands were markedly enlarged in all animals receiving anterior pituitary extract.

Histologically nephrosclerosis or myocardial scarring was prominent in all animals of groups 3 and 4, while no such lesions were observed in any of the others. Twelve of the 13 surviving animals in group 3 showed nephrosclerosis of an average intensity of ++ when graded on a scale ranging between 0 and +++. In the same group 10 of the animals showed some myocardial scarring. In group 4, 7 of the 9 animals averaged++nephrosclerosis while 4 of them showed cardiac lesions. In none of the adrenalectomized rats which received anterior pituitary extract, either alone or in combination with cortin, were there any cardiac or renal lesions.

In order to increase the significance of the observations groups 1, 3 and 8 were repeated under slightly different conditions. There were 20, 20 and 36 animals respectively in the 3 groups. The experiment was terminated after 20 days and only 20 mgs. of L.A.P. were given twice daily. The dose of cortical extract in group 8 was the same as in the preceding experiment. Smaller animals, averaging 85 gms., were used.

Autopsy and histologic examination again revealed that the animals treated with anterior pituitary extract developed marked cardiorenal lesions, as in the preceding experiment, while similarly treated adrenalectomized rats were entirely free of pathologic changes. The results are summarized in Table 2.

CONCLUSIONS

Lyophilized anterior pituitary material induces nephrosclerosis, cardiac lesions and adrenal cortical enlargement in unilaterally

TABLE 1. THE EFFECT OF ADRENALECTOMY ON THE NEPHROSCLEROTIC ACTIVITY OF L.A.P.

Group		No. of Animals		Body Weight		Nephro-sclerosis		Cardiac Lesions		Kidney Weight		Heart Weight		Adrenal Wt.
		Initial	Final	Initial	Final	Inci-dence	Se-verity	Inci-dence	Se-verity	AS % in gms.	AS % of B.W.	AS % in gms.	AS % of B.W.	in mgs.
1	Controls	10	10	152	181	0	0	1	+	1.420	0.78 ± .02	0.758	0.42 ± .02	62
2	Cortin	10	10	152	180	0	0	0	0	1.316	0.72 ± .02	0.759	0.43 ± .02	54
3	L.A.P.	20	13	153	253	12	++	10	+	3.042	1.18 ± .08	1.272	0.50 ± .08	155
4	Cortin and L.A.P.	10	9	152	259	7	++	4	+	2.817	1.08 ± .07	1.147	0.44 ± .01	127
5	Adrenalectomy	10	9	153	182	0	0	0	0	1.272	0.70 ± .09	0.654	0.35 ± .02	—
6	Adrenalectomy and Cortin	10	10	153	182	0	0	0	0	1.380	0.75 ± .05	0.672	0.36 ± .01	—
7	Adrenalectomy and L.A.P.	10	1	153	262	0	0	0	0	2.387	0.91	1.052	0.40	—
8	Adrenalectomy and Cortin and L.A.P.	20	7	153	254	0	0	0	0	2.642	0.87 ± .03	1.161	0.38 ± .02	—

TABLE 2. THE EFFECT OF ADRENALECTOMY ON THE NEPHROSCLEROTIC ACTIVITY OF L.A.P.

Group		No. of Animals		Body Weight		Nephro-sclerosis		Cardiac Lesions		Kidney Weight		Heart Weight		Adrenal Wt.
		Initial	Final	Initial	Final	Inci-dence	Se-verity	Inci-dence	Se-verity	AS % in gms.	AS % of B.W.	AS % in gms.	AS % in B.W.	in mgs.
1	Controls	20	20	79	108	0	0	0	0	.845	0.72 ± .02	.455	0.43 ± .01	38 ± 4.2
3	L.A.P.	20	13	89	155	13	++	6	+	1.674	1.08 ± .01	.856	0.54 ± .01	112 ± 15
8	Adrenalectomy and Cortin and L.A.P.	36	9	82	128	0	0	0	0	1.078	0.84 ± .03	.584	0.47 ± .03	—

nephrectomized rats kept on a high sodium chloride intake—adrenalectomy prevents this effect.

Cortical extract at the dose levels tested does not cause pathologic changes, nor does it inhibit the lesions produced by a simultaneously administered anterior pituitary preparation.

The nephrosclerosis and cardio-vascular damage caused by anterior pituitary preparations is apparently not due to a direct action of the latter upon the kidney and the cardio-vascular system. It is probably mediated by the adrenal cortex, through the secretion of desoxycorticosterone-like compounds. However, our experiments do not exclude the possibility that both pituitary and mineralo-corticoid adrenal hormones are necessary for the development of these lesions.

ACKNOWLEDGMENTS

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EFFECT OF VITAMIN B COMPLEX DEFICIENCY, CONTROLLED INANITION AND METHIONINE ON INACTIVATION OF ESTROGEN BY THE LIVER[1]

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IT IS KNOWN that estrogenic substances can be inactivated by the liver (Zondek, 1935; Israel, Meranze and Johnston, 1937; Golden and Sevringhaus, 1938; Biskind, 1941). When pellets of estrone are implanted intrasplenically in castrated female rats, vaginal cornification will not occur if a normal diet is fed. This is interpreted as being due to the inactivation of estrone by the liver. However, if a diet deficient in the whole vitamin B complex is fed vaginal cornification will occur (Biskind and Biskind, 1942). When yeast was added to the deficient diet the liver regained its ability to inactivate estrone as judged by the return of the vaginal smear to an anestrous condition.

Similar studies were made by Segaloff and Segaloff (1944). They injected estrogens intrasplenically into castrate female rats and noted the per cent showing estrus. They concluded that a deficiency of the vitamin B complex decreased the ability of the liver to inactivate estrone and α-estradiol, and that the addition of thiamine and riboflavin restored the ability of the liver to inactivate these substances. Shipley and György (1944) reported two experiments on the inactivation of estrone. One study was similar to that of the Biskinds in which the absence of the B vitamins produced vaginal cornification and the addition of yeast to the diet restored the power of the liver to inactivate estrone.

The decrease in food intake and loss of body weight may be an important factor in the inactivation of estrogens by the liver when the diet is devoid of B vitamins. In none of the above studies were paired inanition controls utilized, except, in one group of animals receiving α-estradiol and supplements of thiamine, in which caloric intake was restricted (Segaloff and Segaloff, 1944). This present study

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is concerned with the effect of a deficiency of the vitamin B complex and controlled inanition on the vaginal response to splenic implants of estrone. The effect of treatment with methionine was also noted in two experiments.

METHODS

Adult female rats of the Yale strain were used. They received a synthetic diet consisting of: casein (vitamin-free), 18; sucrose, 70; vegetable oil, 8; and salt mixture (U.S.P. No. 2), 4 per cent. An oral supplement of oleum percomorphum was administered twice a week which supplied 500 I.U. of vitamin A and 70 U.S.P. units of vitamin D per dose. A daily oral supplement of crystalline B vitamins was administered which supplied: thiamine, 20γ; riboflavin, 40γ; pyridoxine, 20γ; calcium pantothenate, 100γ; nicotinic acid, 500γ; inositol., 1.0 mgm.; p-amino-benzoic acid, 1.0 mgm.; choline, 5.0 mgm.; and biotin, 0.2γ. The animals were castrated and pellets of estrone averaging 5.0 mgm. were implanted in the spleen. Vaginal smears were made daily and following a control period of anestrous smears the various experiments were started.

RESULTS

Experiment 1. Effect of a deficiency of vitamin B complex and controlled inanition on inactivation of estrogen. Following the control period 24 rats were divided into two groups of twelve. In one group the daily oral supplement of B vitamins was stopped. The other twelve animals continued to receive the supplement of B vitamins each day. They were also paired, according to body weight, with one of the B complex deficient animals and were limited to the amount of food eaten by the deficient animal during the previous day.

The food intake and weight of the vitamin B complex deficient animals steadily decreased, and evidence of failure to inactivate the estrone, as judged by vaginal cornification, appeared at about the 20th day of the deficient diet (Fig. 1). However, the inanition control rats, which were receiving a daily supplement of the B vitamins, showed similar changes in the smears at about the same time. Some variability existed, in that either the vitamin deficient animals or the inanition controls may first show an increase in vaginal cornification. It was concluded that no difference in response existed between the two groups.

Experiment 2. Effect of restricted food intake, B vitamins and methionine on inactivation of estrone. In this study all 13 animals received the daily supplement of B vitamins, but food intake was restricted to 3 grams per day. Seven of the animals received in addition 50 mgm. of methionine per day at the start of the experiment. The methionine treated group lost 55 grams and the group without methionine lost 68 grams in weight during the experiment. The results are shown in figure 2. Five of the six animals restricted to 3 grams of diet per day plus a daily supplement of B vitamins showed estrous reactions. Thus, in the face of inanition alone, the mixture

of B vitamins did not enable the liver to inactivate estrone. The addition of the methionine also did not influence the inactivation of estrone by the liver under the above experimental conditions. The degree and time of onset of the cornification with methionine was not less than that observed in the animals without this substance.

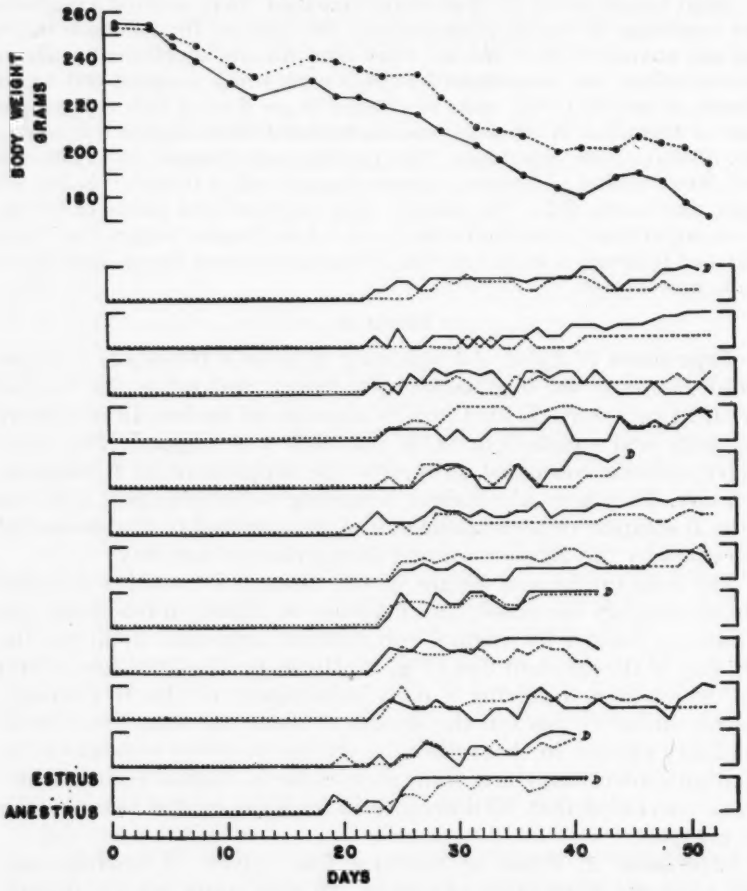

FIG. 1. Effect of a deficiency of the vitamin B complex and-inanition on inactivation of estrone. Solid line represents animals on a vitamin B complex free diet. Broken line represents paired inanition controls receiving vitamin B complex. Days indicate time on deficient diet and paired feeding. D=death of vitamin deficient animal.

Experiment 3. Effect of a deficiency of vitamin B complex and controlled inanition with methionine on inactivation of estrogen. This study, with 19 animals, was similar to experiment 1. Seven rats received a diet deficient in the vitamin B complex. Another seven rats were

paired with them to serve as inanition controls, and received a daily supplement of B vitamins. The last five animals were similarly paired with the deficient rats and received, in addition to the B vitamins, 50 mgm. of methionine per day which was increased to 100 mgm. methionine per day on the 21st day. No difference was found in the onset of vaginal cornification between the three groups (Fig. 3). The

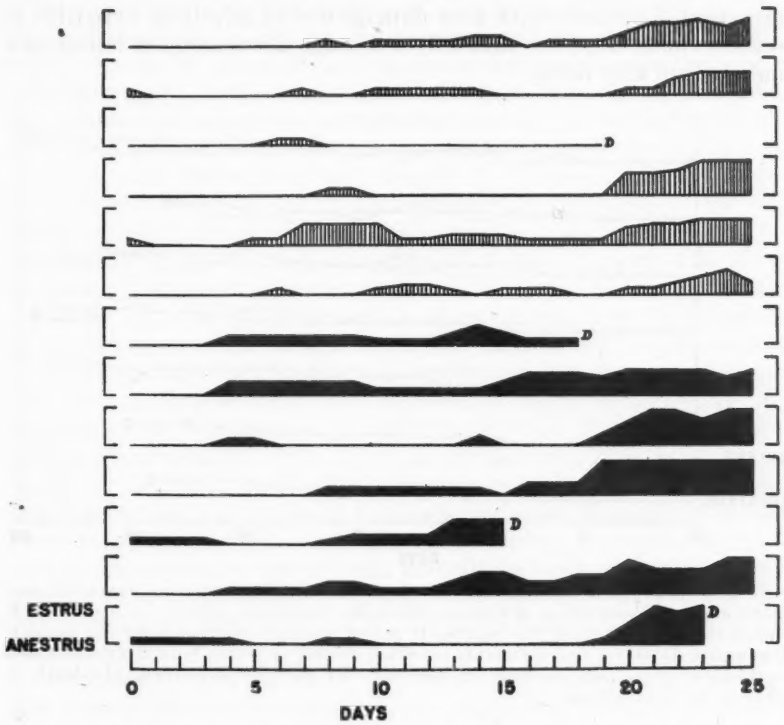

FIG. 2. Effect of restricted food intake, B vitamins and methionine on inactivation of estrone. Crossed-hatched lines represent animals limited to 3 grams of diet per day but receiving a daily supplement of B vitamins. Solid black represents similar animals, but receiving in addition 50 mgm. methionine per day. Days indicate period of restricted food intake.

lack of difference between the deficient rats and the inanition controls confirmed the results in experiment 1. The lack of effect of the methionine, under these conditions, confirmed the conclusion drawn in experiment 2.

DISCUSSION

It is known that the presence of liver damage may produce an upset in estrogen metabolism. With reference to cirrhosis of the liver,

male patients have been reported to show an increase in unconjugated urinary estrogen (Glass, Edmondson and Soll, 1940). This was associated with gynecomastia and testicular atrophy. Other evidence for a change in estrogen metabolism in patients with liver damage has been reported by Wu (1942), Bean (1942) and Perera (1942). Recently Gilder and Hoagland (1946) have reported an increase in the urinary excretion of estrogens and a decreased 17-ketosteroid excretion in patients with liver damage due to infectious hepatitis. It seems evident therefore that if liver damage exists an upset in estrogen metabolism may occur.

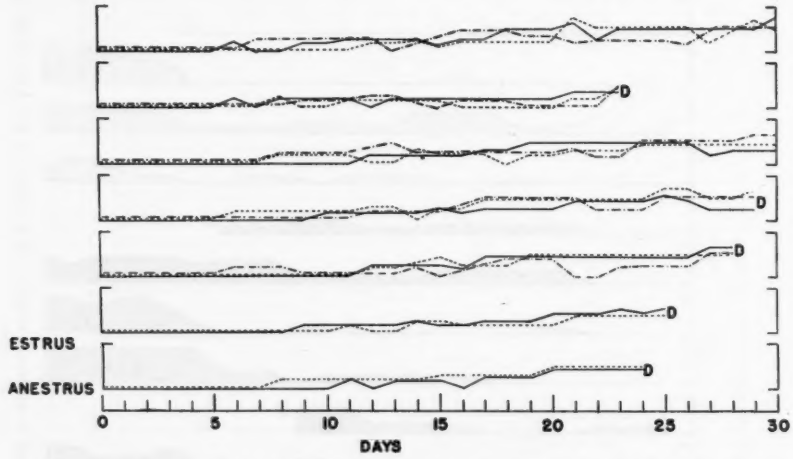

FIG. 3. Effect of a deficiency of the vitamin B complex, controlled inanition, and methionine on inactivation of estrone. Solid line represents animals on a vitamin B complex free diet. Dotted line represents paired inanition controls receiving vitamin B complex. Dash-dot line represents inanition control receiving both B vitamins and methionine. Days indicate time on deficient diet and paired feeding. D = death of vitamin deficient animal.

Experimentally, liver damage and estrogen metabolism has been studied by three means: 1) damaging the liver by toxic agents; 2) acute deficiency of the vitamin B complex; 3) low protein (casein) diets. With reference to the first method it has been shown that the administration of carbon tetrachloride will impair the inactivation of estrogen (Talbot, 1939; Pincus and Martin, 1940).

Utilizing the second method, three groups of workers reported that an acute deficiency of the vitamin B complex interfered with the inactivation of estrogen by the liver. Two of these groups utilized the technique of implanting pellets of estrone in the spleen (Biskind and Biskind, 1942; Shipley and György, 1944). However, no inanition controls were utilized in their experiments. In our present studies, inanition controls, limited to the same amount of food consumed by

the vitamin B complex deficient rats during the previous day, failed to inactivate estrone at the same time as the deficient rats. Thus, the effect of a diet deficient in the vitamin B complex on the inactivation of estrone is not due to a lack of these vitamins *per se*, but is due to the secondary factor of concomitant inanition.

Neither our vitamin B complex deficient animals or inanition controls went into estrus as early as Biskind's rats. Shipley and György (1944) have had a similar experience, but using six rats supplied by M. S. Biskind they obtained results comparable to his earlier experiments. Evidently there is either a strain difference among rats or a difference in the body stores of the B vitamins, particularly thiamine. The addition of yeast or B vitamins to the diet of the deficient animals results in the ability to again inactivate estrone. In such cases, however, food intake and body weight also increases. One can just restrict food intake (Exp. 2), and although the B vitamins are still being supplied, the animals will fail to inactivate estrone.

The paper by Segaloff and Segaloff (1944) is more difficult to interpret. They used castrated rats and noted the incidence of estrus following an intrasplenic injection of estrogens. Two complicating factors seem to be present. First, is the variation in the per cent of animals showing estrus in different groups (five rats per group). Using vitamin supplements that were reported as being ineffective in preventing estrus, the per cent of estrus varied from 60 to 100 per cent, giving a variation of 40%. Thus, is the reported 20% incidence of estrus in thiamine or riboflavin treated rats significantly different from the 60 per cent value of untreated animals? Secondly, the food intake of thiamine treated rats was not restricted, as there was only a loss of 4 grams in weight of such animals as compared with over 30 grams in the other groups. Thiamine with restricted food intake was not tested with estrone but with α-estradiol, but the weight loss was only 9 grams.

Utilizing the third method, the effect of liver damage produced by low casein diets has been studied by a number of workers. Originally one group reported that in the presence of a deficiency of thiamine or riboflavin liver slices failed to inactivate estradiol (Singher, Kensler, Taylor, Rhoads and Unna, 1944). No inanition controls were studied. Later, using a low casein diet the liver slices still failed to inactivate estradiol even though thiamine and riboflavin were being administered (Unna, Singher, Kensler, Taylor and Rhoads, 1944). Other studies have involved the implantation of pellets into the spleen. When such animals were fed a low casein-high fat diet 56 of 91 rats failed to inactivate estrone (György, 1945). Treatment of 8 such animals with 25% yeast in the diet produced a disappearance of the estrus (Shipley and György, 1944). However, this is the equivalent of adding about 12.5% protein to the diet. In the second study supplements of 50 mgm. of methionine, or casein or lactalbumin digest

produced a return of the estrus to the previous anestrous condition whereas a mixture of synthetic B vitamins was without effect (György 1945). It should be remembered that a deficiency of the vitamin B complex and the ensuing inanition is an acute process and does not produce histological changes in the liver, whereas a low casein-high fat diet produces definite changes. Our use of methionine is therefore not comparable to the above experiments of György. Under the conditions of our study methionine was without effect in two experiments (Exps. 2 and 3) in preventing vaginal cornification. This is in contrast to the beneficial effects of methionine when liver damage is produced by a low casein diet, over a long period of time.

In reviewing the above papers it is difficult to explain the reported effectiveness of vitamin B complex in cases of menorrhagia, metrorrhagia, cystic mastitis and premenstrual tension (Biskind, 1943; Biskind, Biskind, and Biskind, 1944). These results on patients do not coincide with experimental data on animals. The use of placebos in evaluating the therapeutic effectiveness of the B vitamins on patients with such menstrual disorders would also be of interest. With respect to a comparison of the clinical and experimental animals studies on the inactivation of estrone the following points must be kept in mind: *I*, Histological liver damage produced in rats does not respond to crystalline B vitamins, but may, depending on conditions, respond to yeast or methionine. If Biskind's patients fall into this group the beneficial effect they observed with B vitamins is at variance with the literature. *II*, The *acute* deficiency of the vitamin B complex in rats (cf. above) and in dogs (Drill, Shaffer and Leathem, 1943) produces a marked decrease in food intake and weight. As the patients were stated to be generally overweight (Biskind, 1943) they do not fall into this group of extreme and *acute* deficiency. *III*, A further factor that should be noted is that in the above rat experiments with pellets the estrogen is not being supplied from a normal source. Animals with pellets in our studies failed to inactivate estrogen after an average loss of 20 to 30 grams in weight. However, if the estrous cycles of thiamine deficient and inanition control rats (without pellets) are studied, estrous cycles cease with an average loss of 12 to 18 grams in weight (Drill and Burrill, 1944). Thus under conditions of *acute* vitamin B complex deficiency one might infer that as food intake and weight decrease the production of estrogen is interfered with before the liver fails to inactivate it. (This inference is made only for acute vitamin B complex deficiency, and not for low casein diets and needs experimental study.) *IV*, Under particular conditions a *chronic* deficiency of B vitamins will produce liver damage, as seen in pellagrins, that does not respond to synthetic B vitamins or methionine, whereas liver extract and dried stomach produces definite improvement (Gillman and Gillman, 1945a; Gillman and Gillman, 1945b; Gillman and Gillman and Brenner, 1945). In a similar liver damage in dogs, there is again a marked decrease in food intake and weight

(Drill and Loomis, 1946). These results differ from those reported on patients by Biskind, both on a change in weight basis, and a lack of effect of crystalline B vitamins on the liver damage in the pellagrins.

SUMMARY

Pellets of estrone were implanted in the spleen of castrated female rats. While being maintained on a normal diet the animals inactivated the absorbed estrone, as judged by the anestrous condition of the vaginal smear. When such animals were placed on a vitamin B complex free diet the ensuing acute deficiency decreased the ability of the liver to inactivate estrone, and the vaginal smears became cornified. Paired inanition control animals, limited to the same amount of food consumed by the deficient animals but receiving B vitamins, also failed to inactivate estrone. Therefore, the effect of acute vitamin B complex deficiency in producing a failure of the liver to inactivate estrone seems to be due to the concomitant inanition. In other animals, limited to three grams of food per day, but receiving B vitamins, a failure to inactivate estrone was also encountered. Supplements of methionine were without effect in preventing the occurrence of vaginal cornification in either the B complex deficient animals or in animals with restricted food intake. The use of the various types of liver damage in studying estrogen inactivation by the liver is discussed as differences exist both in the procedures used and the results.

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THE INFLUENCE OF SELECTED GOITROGENIC COMPOUNDS ON THE THYROID GLAND OF THE CHICK[1]

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THE CHEMICAL nature and activity of more than 300 compounds inhibiting the function of the thyroid gland of the rat have been reported by Astwood (1943) and by Astwood, Bissell, and Hughes (1945). It appeared of interest to extend to the chick observations on a selected group of these agents not only to test their potency in a second species but also as an attempt to obtain a less expensive or more sensitive object for the investigation of antithyroid drugs. An assay in the chick appeared of further interest in view of the extensive hypertrophy which can be induced in the thyroid gland of this species.

METHODS

In general the same methods of assay of potency used in the rat have been followed. The compounds to be tested were mixed in the standard chick feed (Purina) and administered to 3 to 5 day old chicks. After 14 days the chicks were killed, the thyroid glands dissected and weighed, and the iodine content determined. Chicks of this age were used because of convenience and because a trial run indicated that maximal hypertrophy of the chick thyroid in response to 0.5% thiouracil in the diet occurred during the earliest period tested (Table 1).

In studying the uptake of iodine in chicks made goitrous with thiouracil this substance was given mixed in the food in the largest dose well tolerated, and after a period sufficient to allow full depletion of thyroid iodine a solution of potassium iodide was injected subcutaneously and the response in the thyroid iodine content both in regard to dose of iodide and to time after injection was determined.

RESULTS

The data on the antithyroid activity of the compounds tested are summarized in Table 2. In order to compare compounds one with another standard curves were drawn on the bases of the thyroid weights (mg. per 100 gm. body weight) and of the thyroid iodine concentration (mg. per 100 gm. thyroid) in response to increasing amounts of thiouracil in the diet (Fig. 1). The concentrations of

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TABLE 1. 0.5% THIOURACIL FOR 10 DAYS
Effect of Age on Response of Thyroid

Age of Chick Days	Thyroid Weight Mg. thyroid 100 gm. body wt.	Thyroid I_2 conc. Mg. I_2/100 gm. thyroid
5–15	38.4	1.32
15–25	15.3	0.69
25–35	20.8	1.19
Controls	5.4	100.8
35–45	15.6	1.67
Controls	7.8	223.5
45–55	13.0	2.29
Controls	7.6	198

TABLE 2. ACTIVITY OF SELECTED ANTITHYROID AGENTS ON THE THYROID WEIGHT AND IODINE CONTENT OF THE THYROID GLAND OF THE CHICK

Drug	Conc. in Food	Days Treated	No. of Chicks	Daily Body Weight Gain	Thyroid Weight	Iodine Conc.
	%			grams	mg./100 Gm. body weight	mg./100 Gm. wet tissue
2-Thiouracil	0.001	14	4	6.2	5.1	77.0
	0.003	14	3	5.9	6.2	47.5
	0.01	14	3	6.9	11.5	59.1
	0.03	14	6	8.1	22.8	27.1
	0.1	14	3	5.9	51.4	2.7
	0.3	14	4	3.0	51.7	4.2
6-Ethyl-2-thiouracil	0.01	14	3	4.7	32.4	16.0
6-*n*-Propyl-2-thiouracil	0.01	14	5	6.3	52.9	2.3
	0.1	14	3	2.3	44.3	0.65
6-*iso*-Butyl-2-thiouracil	0.01	14	5	4.8	43.2	1.05
6-*sec.*-Butyl-2-thiouracil	0.01	14	3	4.6	41.1	1.30
6-*tert.*-Butyl-2-thiouracil	0.01	14	2	6.5	38.4	1.21
6-Benzyl-2-thiouracil	0.01	14	4	5.0	26.2	1.39
6-*n*-Hexyl-2-thiouracil	0.03	14	4	9.9	5.8	121.
6-Amino-2-thiouracil	0.3	14	3	4.5	7.0	82.2
Thiourea	0.03	14	4	4.4	20.7	26.7
	0.1	14	2	7.1	24.8	1.55
1,3-Diethylthiourea	0.03	14	4	4.9	30.8	9.34
2-Mercaptoimidazoline	0.03	14	4	4.1	67.6	3.19
5,5-Diethylthiobarbituric acid	0.01	14	4	7.3	10.6	51.3
	0.1	14	3	4.7	59.7	12.5
5,5-Dimethylthiobarbituric acid	0.1	14	3	6.2	16.0	9.34
5-Ethyl-5-*iso*propylthiobarbituric acid	0.1	14	4	6.7	57.7	42.1
2-Mercaptothiazoline	0.01	14	4	5.7	12.3	34.4
2-Mercapto-5-aminothiadiazole	0.01	14	4	4.5	11.1	47.8
4-Aminobenzoic acid	0.5	14	4	10.4	9.4	51.2
Sulfaguanidine	1.0	13	4	7.0	8.2	68.3
Sulfadiazine	0.3	14	4	5.6	7.2	75.9
Promizole	1.0	14	2	3.1	14.0	45.3
Controls	0	14	6	7.0	4.8	61.4

thiouracil in the diet are expressed as percentage and these are plotted logarithmically. By means of such curves the antithyroid activity of various compounds may be compared with that of thiouracil when given in the same concentration in the food.

Perhaps the most interesting group of compounds is that in which various substituents have been added to 2-thiouracil at position 6. As in the rat the antithyroid activity in chicks is greatly enhanced when certain alkyl groups and the benzyl group are added to this position. Thus 6-ethylthiouracil appeared about 5 times as active as

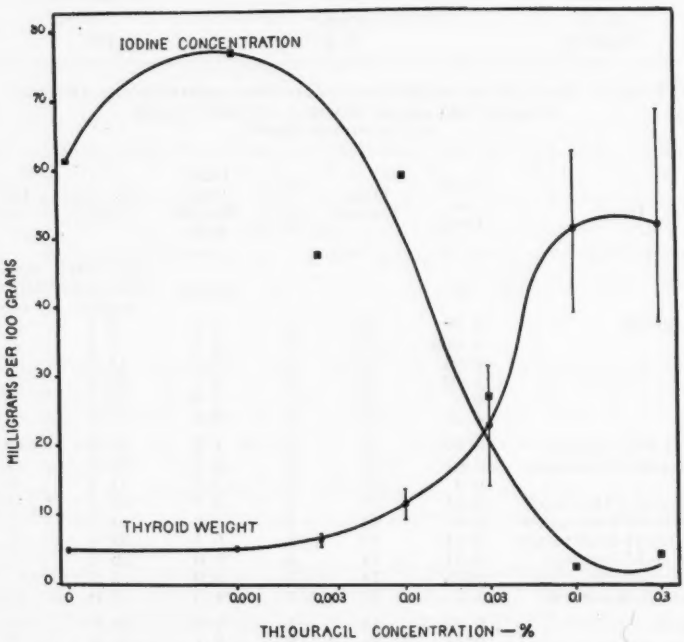

FIG. 1. Dosage-response curves relating the response in iodine concentration and in weight of the thyroid gland to increasing concentrations of thiouracil in the diet. The dose of thiouracil is plotted on a logarithmic scale.

thiouracil whereas 6-*n*-propylthiouracil and the three butyl derivatives tested were about ten times more potent. 6-Benzylthiouracil, although more potent than the parent compound, seemed less active than in the rat. As in the rat, 6-hexylthiouracil and 6-aminothiouracil had almost no activity.

Thiourea in the chick more nearly approached the activity of thiouracil than in the rat, being about 1/3 as active in the chick when administered as 0.1% of the food but of the same order of activity in the concentration 0.3%. 2-Mercaptoimidazoline (ethylenethiourea) was found to have a high activity. Diethylthiourea was not so active

as 2-mercaptoimidazoline but was more potent in the dose tested than thiourea itself.

Of the thiobarbituric acid derivatives thiobarbital (5,5-diethyl-2-thiobarbituric acid) was found to be about as active as thiouracil but not more active as the rat studies, as well as clinical investigations,

TABLE 3. EFFECT OF 0.5% THIOURACIL ON IODINE UPTAKE IN CHICK THYROID

Thiouracil dose	Dose KI μg.	Time after Injection (min.)	Thyroid wt. mg.	Thyroid mg./100 Gm.	Iodine μg.	Iodine mg./100 Gm. Thyroid
0.5% 16 days	500	0	58.4 112.0	66.4 114.4	4.08	2.39
		5	40.6 27.7	40.2 21.5	16.1	23.6
		15	13.6 50.4	17.7 43.5	21.5	33.6
		60	43.9 28.0	53.6 28.9	25.1	35.0
0.5% 25 days	0	60	41.8 112.1	28.8 75.4	0.63	0.41
	10	60	22.4 52.8 76.6	21.6 35.9 48.7	2.96	1.95
	100	60	192.4 56.8	113.8 39.2	27.3	11.0
	10,000	60	136.9 83.0	91.3 58.5	59.0	26.8

Av. wt. at beginning—43 Gm.
Av. wt. Normals at 25 days—236 gm.
Av. wt. 0.5% Thiouracil 25 days—145 Gm.

indicated. 5-Ethyl-5-*iso*propylthiobarbituric acid had similar antithyroid potency and 5,5-dimethylthiobarbituric acid was less potent than thiobarbital.

2-Mercaptothiazoline and 2-mercapto-5-aminothiadiazole in the dosage used appeared to be potent in the same degree as thiouracil.

Contrary to previous reports (Mackenzie and Mackenzie 1943; Astwood, Bissell and Hughes, 1944) certain of the aminobenzene derivatives appear to exert definite antithyroid effect in this species. Thus 4-aminobenzoic acid, sulfadiazine, and sulfaguanidine were found active although to but a slight degree. Promizole was the most active of this series but it is not apparent to which grouping potency

is attributable with this compound as it contains a thiourea-like configuration in addition to the aminobenzene structure.

The data on the effect of 0.5% thiouracil in the diet on iodine up-

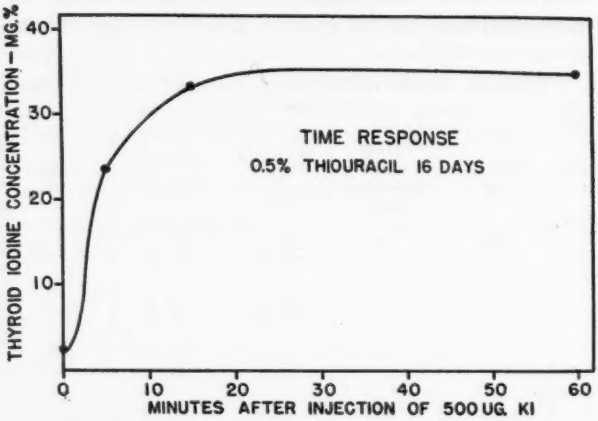

FIG. 2. Time-response curve. Effect of a single subcutaneous injection of 500 micrograms of potassium iodide in 0.5 ml. of water on the iodine concentration of the thyroid glands of chicks treated for 16 days with 0.5% thiouracil in the diet. The chicks were killed at 0, 5, 15 and 60 minutes after injection.

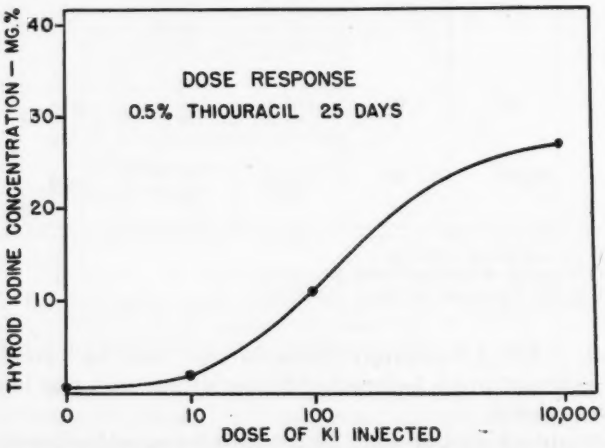

FIG. 3. Dose-response curve. Effect of a single subcutaneous injection of potassium iodide in doses from 0.01 to 10 mg. in 0.5 ml. of water on the iodine concentration of the thyroid glands of chicks treated for 25 days with 0.5% thiouracil in the diet. The chicks were killed one hour after the injection.

take by chick thyroid glands are summarized in Table 3 and graphically presented in Figures 2 and 3. After 16 days of treatment with thiouracil 500 micrograms of potassium iodide were injected sub-

cutaneously and the iodine content of the thyroid gland from 0 to 60 minutes after injection was determined at intervals. In a second experiment 0.5% thiouracil was given in the diet for 25 days. At the end of this period groups of chicks were injected subcutaneously with 10, 100, and 10,000 micrograms of potassium iodide, respectively. Sixty minutes after injection the chicks were killed and the iodine content of the thyroid glands determined.

COMMENT

In the curves used as standard responses to antithyroid agents in the chick which were constructed on the basis of the effect of thiouracil on the size of the thyroid gland and its iodine content it will be noted that in general with increasing doses of thiouracil there is an increase in the weight of the thyroid gland and a corresponding decline in the iodine content. It is readily seen that the increase in thyroid size is less variable than the decline in iodine concentration. Further, with the lowest dosage of thiouracil used a deviation from the general trend occurs. The concentration of 0.001% thiouracil in the diet caused no increase in the weight of the gland and actually increased the concentration of iodine. The explanation, particularly of the effect on the iodine concentration, is obscure. It would be of interest to know if this small amount of thiouracil increased the avidity of the gland for iodine, promoted storage of thyroid hormone in the gland, or acted by some other mechanism.

The figures in Table 2 show that in response to antithyroid agents in the chick the relationship between the degree of thyroid enlargement and the depression in the thyroid iodine concentration is quite variable. The response to the administration of 6-benzylthiouracil provides a good illustration of this, for according to the weight of the thyroid gland this substance would appear about 4 times as active as thiouracil, whereas judging from the fall in iodine concentration it would appear 10 times as active. In general, however, the amount of thyroid enlargement appears to correlate better with observations in the rat than does the fall in iodine concentration.

Under the conditions of this investigation the chick seems to be less suitable for the study of antithyroid drugs than the rat. Facilities for the maintenance of constant temperature were not available and in chicks of this age such a lack imposes a serious limitation. This might have been obviated to a certain extent by using somewhat older chicks although they are less reactive to antithyroid drugs.

The results of the study on iodine uptake are in agreement with those obtained in rats by Astwood (1944–45) and indicate that thiouracil even in large doses does not prevent the prompt accumulation of iodine in the thyroid gland following the injection of potassium iodide. Within 15 minutes of injection the thyroid gland iodine concentration reaches its peak and this is maintained at the end of one

hour. In the doses of 10 and 100 micrograms of potassium iodide between 10 and 20% of the injected dose is present in the thyroid gland at the end of one hour. With the 10 mg. dose of potassium iodide the thyroid gland is probably saturated with iodide and the percentage uptake is quite low.

SUMMARY

A group of selected goitrogenic agents were tested for their relative effectiveness in young chicks. Thiouracil was used as the standard of reference, and its influence on the size of the thyroid gland and on the iodine content when fed for 14 days in concentrations of 0.001% to 0.3% of the food provided the basis for comparison with other compounds similarly mixed in the food. Of the compounds tested the derivatives of thiouracil with certain hydrocarbon groups substituted in position 6 were found most active. Derivatives of 4-aminobenzoic acid were found to have slight but definite activity. The administration of thiouracil in maximal doses for 16 and 25 days did not prevent the rapid accumulation of iodide by the thyroid gland following the injection of potassium iodide.

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INFLUENCE OF ADRENAL CORTICAL STEROIDS UPON THE BLOOD PRESSURE AND THE RATE OF PROGRESSION OF EXPERIMENTAL NEPHRITIS IN RATS

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INTRODUCTION

IN RECENT YEARS it has been demonstrated that long continued administration of desoxycorticosterone acetate (DCA) may induce a rise in blood pressure in patients with or without disease of the adrenal glands (Loeb, Atchley, Ferrebee and Ragan, 1939; Ferrebee, Ragan, Atchley and Loeb, 1939; Thorn, Dorrance and Day, 1942; Engel, Cohn and Soffer, 1942; Roth, Robinson and Wilder, 1943; Perera, Knowlton, Lowell and Loeb, 1944) as well as in normal dogs and rats (Kuhlmann, Ragan, Ferrebee, Atchley and Loeb, 1939; Grollman, Harrison and Williams, 1940; Selye, Hall and Rowley, 1943). In animals treated with DCA, this hypertension is often associated with evidences of renal damage (Selye, Hall and Rowley, 1943; Selye and Hall, 1943; Selye and Pentz, 1943). No similar effect on blood pressure has been reported following prolonged treatment with adrenal cortical extract (ACE), Grollman, Harrison and Williams (1940).

According to Dougherty, White and Chase, ACE has the capacity to enhance the elaboration and release of antibodies (Dougherty, White and Chase, 1944; Dougherty, Chase and White, 1945), whereas these authors failed to observe a rise in antibody titer following the injection of DCA in immunized animals.

The present study was undertaken to compare, in rats, the effect of DCA and ACE upon the blood pressure and velocity of progression of nephritis induced by means of a specific cytotoxic serum, which supposedly establishes renal lesions as a result of an immune response.

EXPERIMENTAL

The animals employed in this study were hooded female rats of the Long-Evans strain, ranging in age from 58 to 68 days. They were fed a modi-

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fication of McCollum's stock diet (Evans and Bishop, 1922) and, in addition to the salt in the diet (1 per cent), they received 0.2 per cent NaCl in their drinking water.

The animals were divided into 6 groups of eight each: Group I served as control; Group II received 2.5 mgs. of DCA a day; Group III, 0.3 cc. of ACE a day; Group IV, anti-rat kidney serum; Group V, anti-rat kidney serum plus 2.5 mgs. of DCA a day; Group VI, anti-rat kidney serum plus 0.3 cc. of ACE a day.

The desoxycorticosterone acetate[1] (DCA) was suspended in peanut oil (10 mgs. per cc.) and 2.5 mgs. injected daily subcutaneously in different areas over the back. The adrenal cortical "lipo-extract"[2] (ACE) was similarly given, the daily dosage being 0.3 cc., which by assay is equivalent to about 1.2 mgs. of corticosterone.

The rabbit anti-rat-kidney serum employed to induce nephritis was prepared as outlined by Smadel (1936). In the present study the serum was given intravenously, starting on the first day of the experiment, for three consecutive days (0.3 cc., 0.5 cc. and 0.7 cc. respectively) to animals in Groups IV, V, and VI. This dosage had previously been shown to induce heavy albuminuria in rats of the same strain.

Systolic blood pressure readings, using the plethysmographic method described by Williams, Harrison and Grollman (1939), were recorded on each rat for 1 to 2 weeks before starting the experiment to accustom the animals to the apparatus, and then at weekly intervals during the course of the study. Readings were made in a constant temperature room at 30°C., and to insure vasodilatation in the rat's tail, an electric light bulb was placed within 1 foot of the apparatus. The blood pressure readings were repeated on each animal until three consecutive determinations agreed within a range of 6 mm. The average of these 3 was taken as the systolic pressure.

Qualitative or quantitative determinations of urinary albumin were made approximately every 2 weeks. Blood urea nitrogen determinations were made at the completion of the study when the animals were sacrificed. Observations were carried on for eight weeks and at the end of this time the animals were weighed, anaesthetized with ether and autopsied. The heart, kidneys, adrenal and thymus glands were weighed and then fixed for histological study. Portions of kidney were frozen and subjected to study for l-amino acid oxidase activity by Dr. David Green.

RESULTS

The animals all grew and gained weight normally during the experimental period and remained well with two exceptions in the group given anti-kidney serum plus DCA; one died in pulmonary edema during the seventh week of observation while in the blood pressure apparatus (tension of 170 immediately before death), and the other had convulsive twitchings on the final day.

Blood pressure: The systolic blood pressure measurements are shown graphically in Chart 1. The middle line represents the average

[1] Desoxycorticosterone Acetate (DOCA) was furnished through the courtesy of Dr. Leo Pirk of Roche-Organon, Inc., Nutley, N. J.

[2] Lipo-Adrenal Cortex was furnished in part through the courtesy of Dr. E. Gifford Upjohn of the Upjohn Laboratories, Kalamazoo, Michigan.

of the weekly readings of all rats in the group, while the upper and lower lines designate the weekly individual extremes. The group which received the anti-rat-kidney serum plus DCA (Group V) developed significant and striking hypertension in contrast to all other groups in none of which elevation in blood pressure occurred.

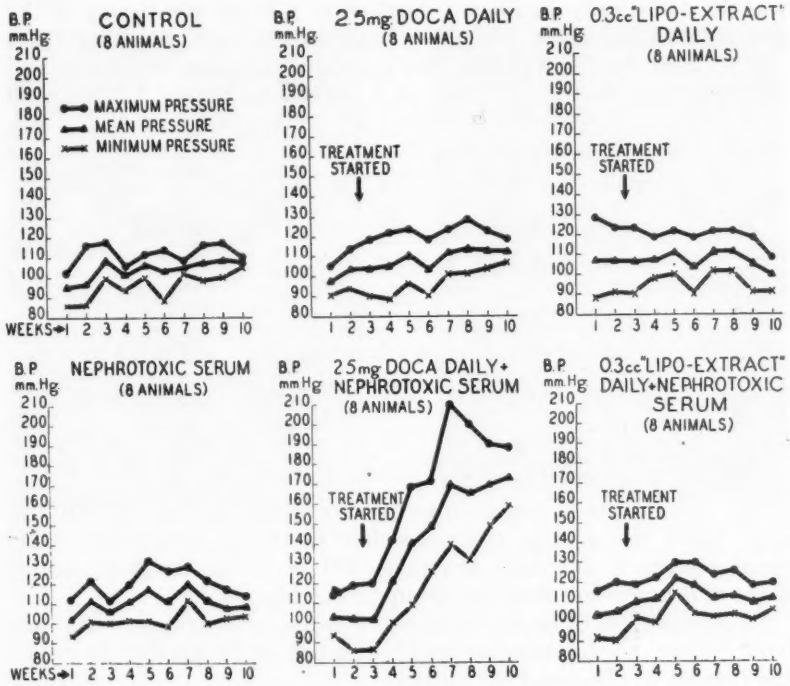

CHART 1
Effect of Adrenal Steroids upon the Systolic Blood Pressure of Normal and Nephritic Rats.
"DOCA" = Desoxycorticosterone Acetate (Roche Organon).
"Lipo-Extract" = Lipo Adrenal Cortex (Upjohn Laboratories).

Albuminuria: Urine specimens, taken at random from the first 3 groups of rats, i.e., those which did not receive anti-rat-kidney serum, revealed an average of 0.4 gram of albumin per liter, a value which is normal for this strain of rats. Similar specimens from the anti-rat-kidney serum injected animals (Groups IV, V and VI) averaged 10.4 grams per liter without significant difference in values from group to group.

Nitrogen retention: No significant retention of urea nitrogen was observed with regularity in any of the groups studied. The maximal value reached in any animal was 32 mg. per 100 cc. This occurred in one animal receiving DCA plus anti-kidney serum.

Organ weights: The average body and organ weights and the range of values are shown in Table 1. It is apparent that the hearts and kidneys of the animals receiving DCA were significantly heavier than those of the control or ACE treated groups.[3] The rats given DCA plus anti-kidney serum, all of which developed hypertension (Group V), showed even more striking enlargement of these organs. There was no statistically significant decrease in adrenal size in any of the groups, although some atrophy was present in 3 rats receiving DCA and in 2 treated with ACE. The individual thymus weights varied widely but, with the exception of 1 animal in Group VI, were all

TABLE I

	Group I Control	*Group II* DCA	*Group III* ACE	*Group IV* Serum	*Group V* Serum + DCA	*Group VI* Serum + ACE
Body weight gms.	202 (194–222)	218 (194–242)	198 (179–237)	200 (188–218)	201* (160–236)	203 (186–230)
Heart mgs.	639 (552–730)	829 (711–923)	669 (551–819)	711 (656–752)	952 (818–1127)	764 (660–919)
Kidneys mgs.	1268 (1109–1589)	1790 (1422–2008)	1373 (1184–1549)	1481 (1335–1603)	1838 (1602–2170)	1592 (1424–1928)
Adrenals mgs.	45.5 (36.4–58.0)	41.4 (32.0–51.7)	43.3 (30.8–55.3)	50.9 (44.7–63)	43.6* (32.5–53.6)	46.9 (36.2–58.7)
Thymus mgs.	214 (169–293)	287 (191–385)	171 (124–263)	274 (215–352)	261* (152–402)	197 (97–260)

* Average of 7 animals.
Figures in parentheses represent range of values.
Figures above those in parentheses represent mean values for each group.

within the accepted normal range for rats of this age and sex. On the other hand, the average thymus weight of the serum injected ACE treated group is definitely less than that of the group given serum alone and much less than the groups receiving DCA.

1-Amino Acid Oxidase: Studies of 1-amino acid oxidase were non-contributory as this enzyme was found not to be present in either the normal or abnormal rat kidneys.

Histopathology: The sections of the kidneys of the 8 animals in the control group (Group I) were essentially normal.

In the DCA injected group (Group II) the kidneys of all the animals showed a definite to marked distention of the majority of cortical and medullary tubules. A peculiar change in the epithelium of many convoluted tubules was present in all animals. There was extreme swelling of many of the epithelial cells in which the nuclei appeared large and vesicular and the cytoplasm vacuolated. This vacuolization was demonstrated not to correspond to lipid deposition in the cells. Such swollen cells exhibited irregular contours with indentations and separation of portions of the cytoplasm. Other tubular epithelia between swollen cells were shrunken, presenting a deeply eosinophilic cytoplasm and pyknotic muclei (Fig. 1). These tubular changes appear quite comparable to those described in animals treated

[3] Statistical analysis of the data was generously done by Dr. C. W. Dupertuis.

with so-called renotropic substances (Selye, Stone, Nielsen and Leblond, 1945), except that in their description there is no mention of atrophic and pyknotic changes in the tubular epithelia. Most of the convoluted tubules which did not show the above morphologic changes were also enlarged and distended. Measurements of cross diameters of tubules showed that the enlargement was not entirely

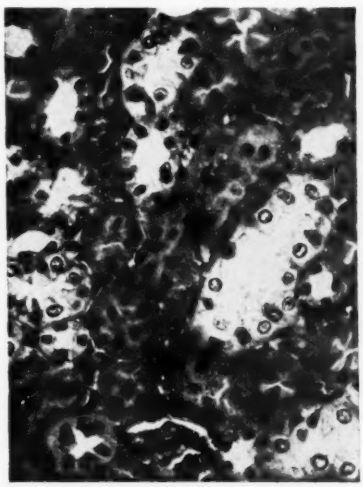

FIG. 1. Kidney of Rat #902 in Group II (DCA) shows distended tubules.

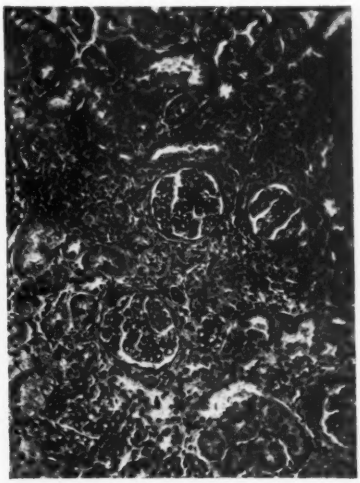

FIG. 2. Kidney of Rat #825 in Group V (DCA plus nephrotoxic serum) shows an area of acute and chronic interstitial inflammation, thickening of basement membrane of glomeruli with increased cellularity of tufts and adhesions between portions of tuft and capsular membrane.

accounted for by the dilatation of the lumen but that an apparent increase in the substance of the tubular wall was a contributing factor. The average diameter of the glomeruli was also increased. This enlargement, however, was less marked than the increase in tubular width. However, no distinct glomerular or vascular lesions were evident. Dry weights of the kidneys were not obtained but there was no histologic evidence of interstitial edema. "Nephrosclerotic" changes as described by Selye et al. after prolonged daily administration of 10 mg. of DCA were not seen, but in the present study only 2.5 mg. of DCA were given daily.

In the ACE injected group (Group III) no distinct renal lesions occurred. In only one animal were there minimal tubular changes somewhat similar to those seen in Group II. Slight glomerular enlargement was noted in some instances.

The kidneys of the animals in Groups IV, V, and VI (animals with

cytotoxic serum nephritis) showed a variety of renal lesions the basic characteristics of which were essentially identical in all of the three groups. The extent and number of these lesions varied within each group, but they were more frequent and more marked in the animals receiving DCA in addition to the nephrotoxic serum. A striking change, to some degree present in most animals of all three groups, was a focal interstitial infiltration mainly by large mononuclear cells.

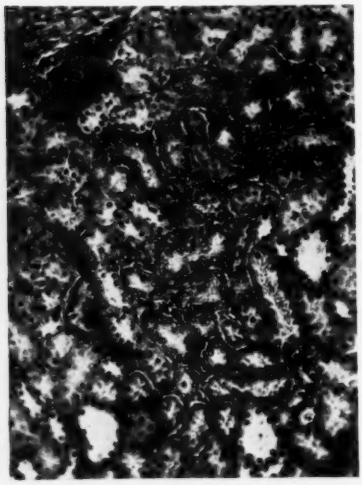

FIG. 3. Kidney of Rat #861 in Group V (DCA plus nephrotoxic serum) shows atrophy of tubular epithelia with increase in number of lining cells.

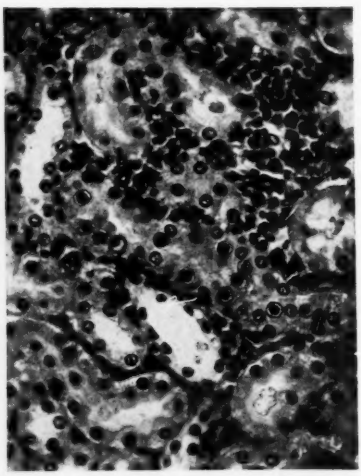

FIG. 4. Kidney of Rat #890 in Group VI (ACE plus nephrotoxic serum) shows large intranuclear inclusions encircled by a distinct halo; also acute and chronic inflammatory infiltration of surrounding interstitium.

Glomerular changes consisting of increase in size and cellularity of the tufts with swelling of the basement membranes were encountered in varying intensity in most of the animals in Groups IV, V, and VI (Fig. 2). Adhesions between glomerular loops and capsule, and crescent formation were seen in some instances. They were most pronounced in about half of the animals in Group V (DCA plus cytotoxic serum). Distinct tubular changes were present in almost all animals injected with nephrotoxic serum. These alterations concerned mainly groups of convoluted tubules, and consisted mainly of atrophy of the tubular epithelia. The atrophic cells, for the area which they occupied, appeared markedly increased in number and had lost the usual cytoplasmic structure. The small amount of ill-defined cytoplasm that remained was pale and slightly basophilic. The nuclei were large and vesicular (Fig. 3). Both glomerular and tubular changes were more intense when they were found in proximity to or surrounded by foci of interstitial inflammation, but interstitial changes and glomerular and tubular lesions were also seen independently. Hyaline and granu-

lar protein casts within distended tubules were found in 4 animals injected with nephrotoxic serum and DCA and in 3 of the 2 other groups. They were present in much larger numbers in Group V. Hyaline droplet changes of the tubular epithelial cells were seen in some of the animals with marked evidence of proteinurea. All the above changes are basically similar but for the most part less advanced than the changes seen by Masugi (1933); Smadel (1937); and Seegal and Loeb (1943) in rats following the injection of an anti-kidney serum. However, in this series, the focal interstitial infiltration was more striking than is apparent from the descriptions of the above authors. Besides these findings, the convoluted tubules in the animals in Group V showed changes identical with those seen in Group II (normal rats receiving DCA).

Perivascular infiltrations in the kidney by acute or chronic inflammatory cells or both were observed in 5 animals of Group V; in 2 they were extremely marked. Similar lesions, moderately severe in nature, were also seen in 2 of the animals injected with nephrotoxic serum alone and also in one animal that received cortical extract and no nephrotoxic serum. In no instance was there involvement of the vascular wall itself. These lesions therefore could not be classified as true periarteritis. One animal of Group V showed rather intense proliferation of endothelial cells within small arteries. Moderate but similar changes were found in several others of this group. No changes were demonstrable in the elastic elements of the vessels in any of the animals. Small plaques of intimal thickening composed of cellular fibrous tissue were seen in several instances in animals of Group V, but in none of the others. In 5 animals of Group V, there was present a more or less diffuse and essentially chronic inflammatory infiltration of the renal pelvis with the formation of small calcium concretions in 2.

Atrophic tubular cells in 1 of the animals in Group IV and in 3 in Group VI showed intranuclear inclusions similar to those described by Farber and Wolbach in the kidneys and other organs of infants (Fig. 4).

In sections of the myocardium, the muscle fibers appeared hypertrophied in all cases where there was gross enlargement.

The adrenals in most of the animals treated with DCA and in several injected with ACE, showed atrophy of the cortical cells with loss of lipid, as demonstrated by osmic acid impregnation. However, in some instances, atrophic changes were also seen in animals of Group IV (anti-kidney serum without DCA or ACE). The morphology and the distribution of atrophic changes did not differ among animals injected with DCA or ACE, nor did they always parallel the extent of gross atrophy.

DISCUSSION

Desoxycorticosterone acetate, in dosages of 2.5 mg. a day, induced no hypertension in rats in the course of 8 weeks, but when this same

dose was given to animals with cytotoxic serum nephritis, striking hypertension resulted in every instance. This is evidently not the result of the cytotoxic serum alone because no such blood pressure changes were seen in the rats receiving only this or even in those animals given ACE as well. The mechanism involved in the development of this hypertension in DCA treated nephritic rats remains wholly obscure. It is possible that it is a reflection of the more advanced renal lesions observed in this group of animals. However, the degree of hypertension cannot consistently be correlated with the extent of renal damage. Another possibility is that DCA influences the renin mechanism and that this is therefore more apparent in rats with nephritis than in normal animals. It is also conceivable that hypertension developed for some reason in association with sodium retention as demonstrated in dogs and rats receiving DCA (Kuhlmann, Ragan, Ferrebee, Atchley and Loeb, 1939; Harkness, Muntwyler, Mautz and Melors, 1942; and Clinton, Thorn, Eisenberg and Stein, 1942). This explanation would be compatible with Selye's findings that DCA alone, even in four times the dosage employed in the present study, produced no significant hypertension, but when given in conjunction with 1 per cent NaCl in the drinking water (five times the concentration used in this experiment) the hypertensive effect became striking (Selye, Hall and Rowley, 1943). Finally, it may be said with certainty that the observed hypertension was not related to the elaboration of pressor amine by l-amino acid oxidase since this enzyme was not found in the kidneys of either the normal or hypertensive rats.

Significant increases in weight of the heart and kidneys were observed in all rats injected with DCA. Since this was true of those animals in which no blood pressure changes occurred (Group II receiving DCA alone), the increase was patently not secondary to the development of hypertension, although, where hypertension existed (Group V) the enlargement of the organs was even more marked. It does not seem likely that the increased weights recorded in the DCA treated rats was due to an accumulation of interstitial fluid, although to establish this with certainty dried organ weights would be essential. There was no edema evident grossly, no increase in intercellular material was noted microscopically and where the kidney tubules were measured, they were found actually larger in the DCA groups than in the others, this being particularly true in areas showing the "specific" tubular changes.

Neither the increase in organ weights, nor the renal tubular lesion seems to be a specific response to DCA alone. In the present study, slight cardiac enlargement was noted in rats receiving anti-kidney serum plus ACE and it is possible that with larger doses of this "lipoextract" more hypertrophy would be evident. Furthermore, Selye reports kidney enlargement and, in fact, very similar microscopic renal tubule changes (i.e., many tubules showing a distention with marked vacuolization of the epithelial lining) following the administration of

methyl testosterone, thyroxin or anterior piuitary extract, all of which he terms "renotropic" substances (Selye, Stone, Nielsen and Leblond, 1945). The fact that the DCA changes reported in the present study appear "renotropic" rather than "nephrosclerotic" as reported by Selye may result either from the smaller daily dose of the steroid used in the present study or from the lower intake of NaCl, since without the addition of salt, Selye observed only minimal changes.

The mechanism for the origin of chronic glomerulonephritis, experimentally produced or occurring naturally, is unknown. Masugi (1933) working with a duck anti-rabbit kidney serum was able to produce chronic glomerulonephritis in rabbits and suggested that the mechanism for its production was allergic in nature. More recently Kay (1940) likewise using a duck anti-rabbit-kidney serum, has produced a similar nephritis and has advanced the theory that the nephritis which ensues results from the interaction of antibodies produced in response to the injected duck protein which acts as an antigen.

In the case of cytotoxic nephritis in the rat, although the process is initiated promptly by the introduction of the preformed antibodies, it seems possible that the progressive nature of the lesion may be due to subsequent production of antibodies to modified autogenous kidney antigen. With this in mind, it is conceivable that treatment which stimulates antibody production might be expected to enhance the nephritis. Dougherty, White and Chase, as previously mentioned, have presented evidence in support of the enhancement of antibody production by adrenal cortical extract. Therefore, an acceleration of the cytotoxic nephritis in the ACE treated Group VI was considered a possible eventuality. However, neither the course nor the histological findings indicated a difference in the rate of progression of the nephritis in this group as compared with that in Group IV injected with serum alone. Since there was only slight evidence of lymphatic tissue atrophy in Group VI, as indicated by thymus gland weights, the dosage of adrenal cortical extract may have been insufficient to increase antibody production. On the other hand, the animals of Group V treated with DCA and serum presented renal lesions more marked in degree and extent than either Group IV or VI, and yet the limited data available suggest that DCA does not stimulate the release of antibodies.

SUMMARY

A comparison has been made between the effects of DCA and of ACE in rats upon the blood pressure and the rate of progression of nephritis induced by a cytotoxic serum. Comparative effects of DCA and ACE were also studied in normal animals. Striking hypertension developed in nephritic rats following the administration of DCA. There was no observed rise in blood pressure in non-nephritic rats given the same doses of DCA, nor in nephritic animals which did not

receive DCA. The dose of ACE employed failed to induce hypertension in either the nephritic or normal rats. DCA caused renal and cardiac enlargement in both normal and nephritic rats, although the degree of enlargement was greater in the latter group which exhibited hypertension. DCA caused a specific renal lesion in the tubules of both normal and nephritic animals. These changes were not found in most of the animals treated with ACE. Contrary to expectation, the nephritis in the rats receiving DCA and cytotoxic serum was more intense than in animals receiving cytoxic serum alone or cytotoxic serum and ACE. The mechanism responsible for the development of hypertension and augmentation of the nephritic process by DCA remains obscure.

ACKNOWLEDGMENTS

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HYPERPLASIA OF THE THYROID GLAND OF RHESUS MONKEYS AFTER THIOURACIL TREATMENT

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ADMINISTRATION of thiouracil to rhesus monkeys (Macaca mulatta) causes hyperplasia and colloid depletion in monkeys as it does in other animals and in man. Certain special histological features of thyroid hyperplasia in the monkey are described in this report.

Changes in some physiological constants in surgically induced hypothyroid monkeys have been described previously by Fleischman, Shumacker, and Wilkins (1945) and by Jailer, Sperry, Engle, and Smelser (1944).

The effect of thiouracil on serum cholesterol, circulation times and white blood cell count of rhesus monkeys was reported by Aranow, Engle, and Sperry (1946). In these studies thiouracil was administered orally to four adult female monkeys in increasing dosage over a period of 14 months. Thiouracil was given daily except Sunday. On Saturday a double quantity was given. The quantity administered was 0.1 gm. for the first 28 days, 0.2 gm. for the next 61 days, and 0.4 gm. for an additional 76 days. Since no demonstrable physiological effects were noted during this period of 165 days, treatment was stopped, and a period of 36 days with no treatment ensued. After this interval, treatment was reinstituted with increased dosage, 0.8 gm. of thiouracil being administered daily, 6 days per week, with no treatment on the 1st day of the week. (Total weekly oral dose—4.8 gm.) Thiouracil was administered at this level until the conclusion of the experiment. (173 to 243 days).

Two animals received a potent thyrotrophic preparation in addition to thiouracil for 8 and 12 days immediately preceding autopsy.

RESULTS

Biopsies of the thyroid gland were taken at intervals during the treatment and the entire gland was examined at autopsy on 3 monkeys. A summary of the details relating to the biopsies is given in Table 1. Weights of the glands were not taken since adequate control data for thyroid weights were not available.

Biopsies were taken from two monkeys, 694 and 605 after 28 days

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on 0.1 gm. thiouracil daily, and 57 days on 0.2 gm. per day. The total quantity was 14.2 gms. over a period of 85 days. The thyroid follicles were uniform in shape and configuration, and were full of colloid. The epithelium was cuboidal or low columnar. There was no evidence of hyperplasia in 694, but 605 showed minute colloid-containing vesicles

TABLE I. THYROID RESPONSE TO THIOURACIL

Thiouracil Treatment	Thyroid Examination	Structure of Thyroid
0.1 gm.—28 days 0.2 gm.—61 days Total 15.0 gm.—89 days	Biopsy 694 605	No change; normal thyroid.
0.4 gm.—79 days 31.6 gms.	No biopsies	
No treatment 36 days		
0.8 gm.—73 days 6×weekly	Biopsy 788	Slight change. High columnar epithelium. Colloid normal.
50.4 gm.	784	Tall columnar epithelium. Intrafollicular vesicles. Colloid present in all follicles.
0.8 gm.—173 days 6×weekly 118.0 gm.	Biopsy 694 784	Marked hyperplasia. Colloid sparse. Follicles irregular in shape. Many intrafollicular vesicles.
0.8 gm.—206 days 6×weekly 141.6 gm.	Biopsy 605 784	Extreme hyperplasia. Follicles irregular in shape and size, many without colloid. Secondary intrafollicular vesicles.
No treatment, 49 days	Biopsy 605	Almost complete recovery. Low cuboidal epithelium, follicles full of colloid.
No treatment, 34 days	Autopsy 784	Almost complete recovery, slight residual hyperplasia.
0.8 gm.—239 days 179.2 gm. Thyrotrophic hormone terminal 8 days.	Autopsy 694	Extreme hyperplasia, with 3 mm. cystic follicles. Follicles irregular, none with normal colloid.
0.8 gm.—243 days 182.4 gm. Thyrotrophic hormone, terminal 11 days.	Autopsy 788	Extreme hyperplasia, follicles large, irregular, none with normal amount of colloid.

within the epithelial surface of the follicles. Biopsies on two monkeys (788 and 784) were taken after having received 0.8 gm. per day for 73 days (total 50.4 gms.). The structure of the thyroid gland of monkey 788 was somewhat modified. The epithelium was of a high columnar type. The contour of the follicles was uniform and not irregular in shape. There was little infolding of the follicle wall. The follicles were full of normally staining colloid.

The structure of the thyroid gland of monkey 784, which had received the same treatment as had 788, was profoundly modified. In this animal there were a large number of intrafollicular vesicles, two of which are shown in Figures 1 and 2. These structural modifications of a hyperplastic thyroid gland have not been described in other animals or in man. They were found in this animal and uniformly occurred in the other monkeys at subsequent biopsies. Although the

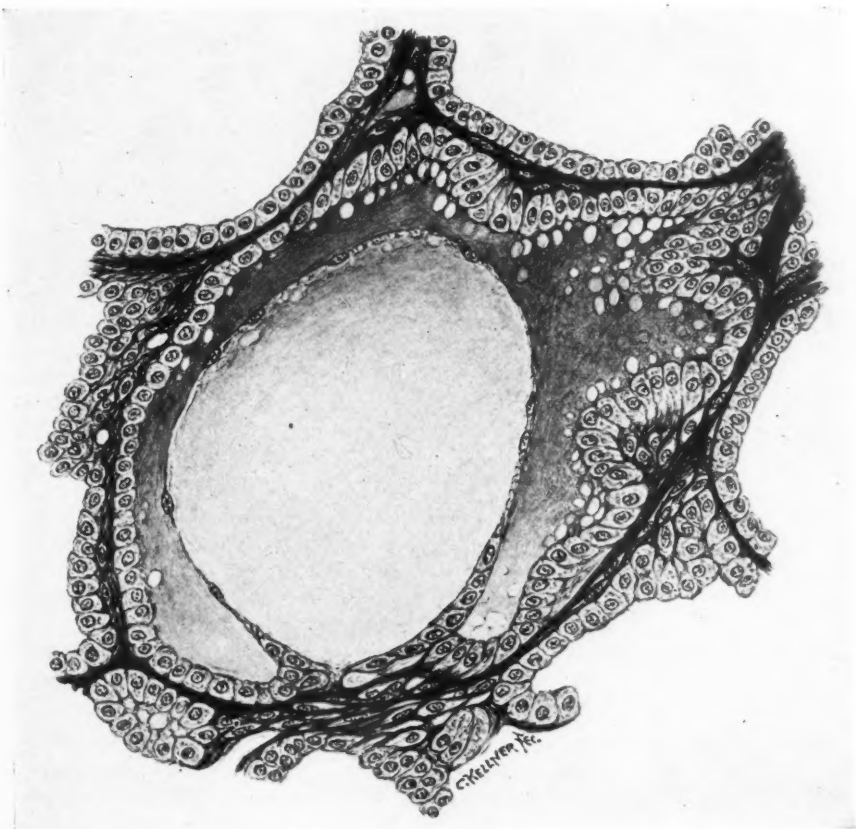

FIG. 1

histological structure is described for one monkey only, the same structures appear in the other animals at later stages in the hyperplasia of the gland.

The follicles of this gland (784) were highly irregular in form and configuration. The epithelium was low columnar or cuboidal in some areas, and high columnar in others. Where the epithelium was high columnar, invaginations into the follicle occurred. These were small

intraepithelial cysts of large colloid-containing vesicles within the follicle (Fig. 1). The epithelial cells at the base of vesicle were high columnar. The shapes of the cells then changed progressively to low cuboidal and over the apex of the vesicle were flattened squamous cells with very thin, attenuated cytoplasm. These vesicles were very numerous. A study of serial sections revealed that each vesicle had developed from the follicular epithelium, and was attached to the

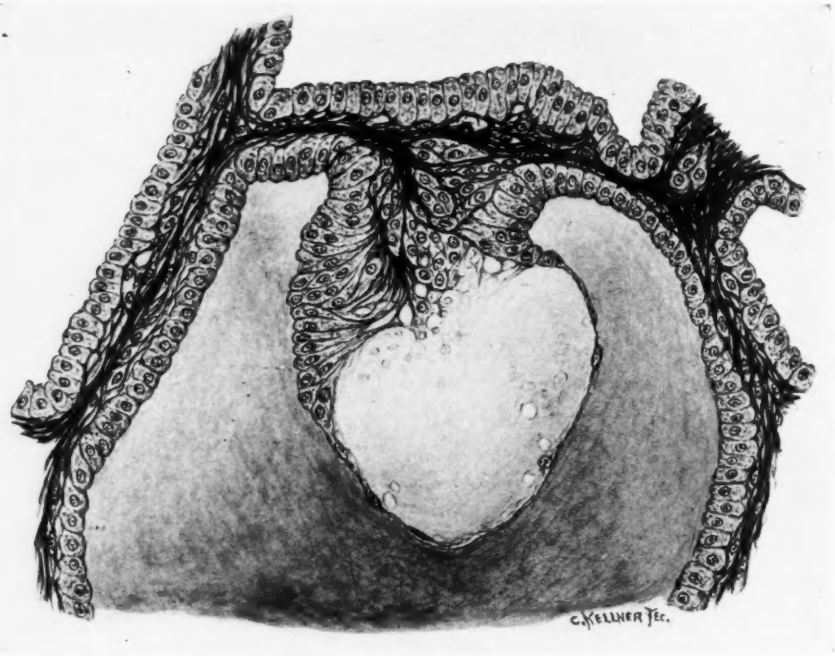

FIG. 2

follicle wall by a single pediment. Some larger follicles had three distinct and independent intrafollicular vesicles, which had developed as invaginations from the follicular wall. The surface of the vesicle was completely covered and delimited by an intact epithelial membrane. There was apparently no hiatus in the epithelial covering, and no communication between the intravesicular cavity and the lumen of the follicle. There was no visible stromal supporting tissue of the vesicle nor distinct basement membrane which could be demonstrated by Masson's trichrome stain. Reticular fiber preparations were not made.

The intrafollicular vesicle contained colloid, which stained less deeply than the colloid of the follicle. It is not known whether the

colloid was secreted by the cells into the vesicle or whether there was any diffusion or other interchange of colloid between vesicle and follicle.

The follicle shown in Figure 2 is characterized by the very high epithelium at the base of the vesicle. In this follicle is seen also the collagenous stroma which has followed the infolding of the epithelium into the pediment.

Further biopsies of the thyroid glands of two animals (694 and 784) were made after the animals had received 0.8 gm. of thiouracil, 6 days each week for 173 days (total 118.0 gm.). There were marked histological evidences of hyperplasia. The follicles were very irregular in size and shape, and many were almost devoid of colloid. Those follicles on the periphery of the gland were distended and irregular in contour but contained colloid. The staining properties of the colloid varied considerably. In many hyperplastic follicles no colloid could be seen in slides stained with hematoxylin and eosin but was demonstrated by Masson's trichrome stain.

Two additional biopsies were made on monkeys 605 and 784, after 206 days on 0.8 gm. thiouracil, six days weekly (total 141.6 gms.). Extreme hyperplasia of the thyroid gland was observed at this time. Follicles were irregular in size and configuration, and many were without demonstrable colloid. The epithelium ranged from high to very high columnar. Nuclei in the tallest cells were oval and were located near the apex of the cell. There were some secondary interfollicular vesicles.

Further biopsies were not done. Two monkeys, 784 and 605, were kept without treatment of any kind for 34 and 49 days respectively, at which dates the animals were sacrificed and autopsied.

The thyroid gland of monkey 784, which had lived for 34 days with no treatment showed some recovery from the condition of extreme hyperplasia which was observed at the last biopsy described above. There were some residual structural changes suggestive of hyperplasia. The follicles were somewhat irregular in shape. The epithelium was cuboidal, the cytoplasm indistinct, the nuclei pycnotic. Remnants of shrunken or collapsed intrafollicular vesicles were present.

The thyroid gland of monkey 605 which had been without treatment for 49 days showed a greater degree of recovery from the extreme hyperplasia observed at the last biopsy. The follicles were of uniform shape and configuration. The irregularity of contour previously observed had disappeared. All follicles were uniformly filled with colloid. The epithelium was characteristically low cuboidal. There were, however, numerous follicles which gave evidence of a previous intrafollicular haemorrhage. Masses of pigment, probably haemosiderin, within and around the follicular epithelium were present.

Recovery from the thiouracil-induced hyperplasia was apparently complete as determined histologically. The gross size of the gland seemed to be no greater than that of an untreated monkey of the same weight. The extent of injury, and the cause of the extensive intrafollicular haemorrhage could not be determined.

Two of the monkeys (694 and 788) were given a thyrotrophic hormone preparation concurrently with 0.8 gm. thiouracil daily as a terminal treatment. The first monkey was treated for 8 days; the latter for 11 days. The result of the administration of both thiouracil and thyrotrophin was to intensify the degree of structural hyperplasia. Following this treatment, the cystic follicles were highly variable in size, and ranged from minute to cystic follicles which were 3 mm. in diameter. There were no follicles with normal colloid content, and some were almost devoid of colloid. The epithelium was uniformly very high columnar. The nuclei were either round or oval, and varied from a central to an apical position in the cell. The secondary intrafollicular vesicles were very numerous.

SUMMARY

Hyperplasia of the thyroid gland of the monkey did not occur after treatment with 0.1 and 0.2 gm. of thiouracil daily for 12 weeks. The first biopsy which was taken after administration of 0.8 gm. daily for 73 days showed extreme hyperplasia of the thyroid gland. All subsequent specimens obtained after longer periods of treatment showed severe hyperplasia. One or more intrafollicular vesicles within a hyperplastic follicle were found to be characteristic of thyroid hyperplasia in these monkeys. The hyperplastic thyroid returned toward normal within 34 days after cessation of thiouracil treatment, and by the 49th day the thyroid gland was histologically normal in shape, colloid content and height of the epithelium.

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SOME EFFECTS OF THE ADMINISTRATION OF THIOURACIL TO MONKEYS[1]

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THE GOITROGENIC effects of thiourea, the sulfonamides and related compounds such as thiouracil have been well known since the reports by the Mackenzies (1941, 1943) and the extensive investigations of Astwood and associates (1943 et seq.). The action of thiouracil in the rat and other lower forms has been discussed by many investigators. The potential therapeutic value of the drug has led to numerous and careful investigations of its action in man (Astwood 1943, 1944 et al.). For further experimental study the adult rhesus monkey (Macaca mulatta) was chosen. Other studies on the effect of hypothyroidism in these animals have been reported from this laboratory (Jailer, Sperry, Engle and Smelser 1944; Engle 1944) and afford several measurements of thyroid function which were useful in this investigation.

METHODS AND MATERIALS

Four adult female rhesus monkeys, each of which had been under observation in the laboratory for at least a year, were chosen. The animals were fed their normal laboratory diet, consisting of fruits, vegetables, bread, grain, rice and occasionally eggs. The monkeys weighed 4.0, 4.4, 5.0 and 5.7 kg. respectively at the beginning of these experiments.

At first, weights were determined at weekly intervals, but when it became apparent that significant changes were not occurring, the interval was increased.

Circulation times were measured by the sodium cyanide method as used previously by Jailer et al. (1944). From 0.07 cc. to 0.12 cc. of a 1% solution of NaCN in saline were injected into the posterior popliteal vein and the interval between injection and the gasp response was timed with a stopwatch calibrated in 0.1 second. One control animal was used for circulation time and cholesterol determinations to evaluate any possible effect of NaCN injection.

Cholesterol estimations were made on venous blood drawn in the postabsorptive state, by the method of Schoenheimer and Sperry (1934) as modified by Sperry (1945).

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Biopsies of the thyroid glands of the treated animals were taken at intervals, and the specimens sectioned and examined microscopically. Complete autopsies were performed on three of the four animals. The histological changes in the thyroid gland are reported by Engle and Aranow (1946).

Two of the animals (788 and 694) received daily intramuscular injections of 0.2 gm. thyrotrophic hormone[2] for eight and eleven days, respectively, immediately preceding autopsy.

Studies of the formed elements of the blood were made on freshly drawn venous blood aspirated directly from a needle inserted into the posterior popliteal vein. Counts and studies of blood smears were made by the Division of Hematology of the Department of Medicine.

Control determinations were made of weight, serum cholesterol, and circulation time before medication began. Thiouracil[3] was given orally in tablet form. The tablets were placed on the posterior portion of the dorsum of the tongue, and a small amount of water was administered. The oropharynx was inspected after deglutition in order to be assured of ingestion of the tablets. With practice it was possible to give as many as four tablets at a time. On January 31, 1944, each animal was started on 0.1 gm. of thiouracil daily. This dosage was chosen because on a kilogram body weight basis, this amount is roughly equivalent to the largest amounts which were then being used in the treatment of human thyroid disease. This was the smallest tablet in which the drug was then being dispensed. After four weeks, when neither toxic nor measurable metabolic effects were evident in any of the monkeys, the dose was increased to 0.2 gm. per day.

On April 27 the dosage was again doubled, and for the next seventy-seven days 0.4 gm. was administered daily in the form of two 0.2 gm. tablets. Since no metabolic effects were noted by the end of this period, all treatment was suspended from July 13 through August 20. When the daily dosage was 0.4 gm. or less, the entire dose was given each morning before the regular feeding. No drug was administered on Sundays, and a double dose was given on Saturday mornings. During the later period of the experiment when 0.8 gm. was given each day, 0.4 gm. was given morning and afternoon, with a single 0.8 gm. on Saturday morning, and none on Sunday.

From August 21 to the conclusion of the experiment 0.4 gm. was administered twice daily (0.8 per day) six days each week.

Animals 694 and 788 received 0.8 gm. of thiouracil each for 36 weeks until sacrificed on May 1 and 4, 1945, respectively. On April 22 these two animals received 200 mg. of thyrotrophic hormone and this was repeated daily until the animals were killed eight and eleven days later.

Blood counts were made on all animals on November 11, 1944, after $11\frac{1}{2}$ weeks on 0.8 gm. of thiouracil daily. Beginning on December 15, weekly counts were made as long as thiouracil was given.

When it was evident that this treatment had a deleterious effect on the white cells, two monkeys, 694 and 788, were given pyridoxine and folic acid concentrate at different times. Thiouracil administration continued during each of these periods of treatment.

[2] This material, prep. 33A-4, was prepared by Dr. George Smelser, Department of Anatomy, College of Physicians and Surgeons, to whom we are greatly indebted.

[3] The thiouracil (Deracil Lederle) and the folic acid concentrate used in these experiments was kindly supplied by Lederle Laboratories, through the courtesy of Dr. B. W. Carey.

RESULTS

During the experiment, there was no significant change in the weight of any animal; during thirteen months two animals gained 6% in weight; another 2% and the fourth lost 2%.

Cholesterol

The total serum cholesterol concentration fluctuated widely, but there was no significant trend in either direction. A typical example is shown in Figure 1 in which the record of monkey 605 is plotted.

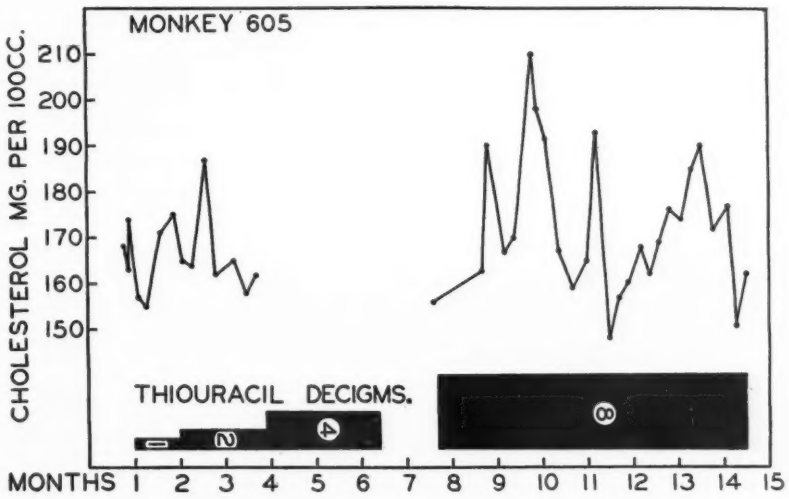

FIG. 1

The fluctuations were larger and more abrupt than were observed in our previous studies on monkeys in which the serum cholesterol concentration usually was maintained within a fairly narrow range of variation, or changed slowly on a fairly smooth curve during the periods between experimental procedures. Although too few data are available on the behavior of serum cholesterol over extended periods of time in normal monkeys to justify a conclusion, the results indicate that thiouracil had the effect of "labilizing" the mechanism which controls the serum cholesterol concentration.

At each of the determinations, of which there was a total of 164, the free cholesterol concentration was also determined. The findings were in accord with evidence previously reported, indicating that the proportion between the cholesterol fractions of the blood serum is not controlled by the monkey with the rigidity which characterizes the human being. No noteworthy effect on the ratio between combined and free cholesterol resulted from the administration of thiouracil, even at the largest dosage of 0.8 gm. daily.

Circulation Time

In three of the animals whose circulation times were within the "normal" range (Jailer et al. 1944) before treatment, there was a general tendency for the circulation time to be lengthened by thiouracil. In the fourth animal, the initial circulation time was considerably prolonged, and did not change significantly during the experiment. The actual significance of changes in circulation time is slight.

TABLE I. WHITE BLOOD CELL STUDIES ON MONKEYS TREATED WITH THIOURACIL
(First value obtained after a minimum of 80 days treatment with 0.8 gm.)

Date	Total WBC	Differential Counts (%) PmnN	Eos	Bas	Lymph	Mono	Total PmnN
		Monkey #694					
12-15-44	6500	22	0	0	78	0	1430
12-21-44	6300	33	0	0	63	4	2079
12-28-44	4200	17	0	0	80	3	714
1- 5-45	6800	24	0	0	71	5	1632
1-12-45	4300	19	0	0	79	2	817
1-18-45	4500	13	0	0	84	3	585
1-25-45	5300	12	0	0	87	1	636
2- 2-45	3500	9	0	0	85	6	315
2- 9-45	4600	18	0	0	77	5	828
2-15-45	6400	27	0	0	68	5	1828
2-23-45	8400	37	0	0	54	13	3408
3- 2-45	9450	40	0	1	57	2	3780
3- 9-45	5000	17	0	0	72	11	850
3-16-45	7800	19	1	0	76	4	1482
3-22-45	5500	18	0	0	70	12	990
3-27-45	7800	18	2	0	73	7	1404
4- 3-45	4800	17	4	0	68	11	816
4-10-45	4800	17	12	0	70	1	816
4-18-45	9200	13	9	0	71	7	1196
*							
4-30-45	10900	51	7	1	31	10	5559
		Monkey #788					
12-15-44	6300	18	0	0	81	1	1134
12-21-44	7100	34	5	0	55	6	2414
12-28-44	6200	23	0	0	74	3	1426
1- 5-45	8200	18	0	0	78	4	1476
1-12-45	8000	47	0	0	44	9	3660
1-18-45	6900	22	0	0	78	0	1518
1-25-45	6800	23	0	0	72	5	1564
2- 2-45	5900	28	0	0	72	0	1702
2- 9-45	7300	15	0	0	85	0	1095
2-15-45	6500	30	2	0	66	2	1950
2-23-45	6100	32	0	0	62	2	1952
3- 2-45	6000	16	0	0	80	4	960
3- 9-45	6300	27	1	0	67	5	1701
3-16-45	8200	23	0	0	73	4	1886
3-22-45	7800	27	0	1	70	2	2106
3-27-45	8600	20	1	1	75	3	1720
4- 3-45	4200	26	0	0	70	4	1092
4-10-45	5300	34	0	0	60	6	1802
4-18-45	9200	24	1	1	69	5	2208
*							
4-30-45	13100	55	1	0	35	9	7205
5- 4-45	14500	53	0	0	38	9	7685

* Beginning on 4-23-45 and continuing until animal was sacrificed, daily intramuscular injections of thyrotrophic hormone were given.

Blood Counts

The problems of determining the "normal" blood count of rhesus monkeys from this laboratory have been fully discussed by Crafts (1941). Three of the four animals showed total white blood counts below the lowest "control" value reported by Crafts at the first examination. The counts for two monkeys are shown in Table 1. All of the animals had received 0.8 gm. of thiouracil, six days each week, for a minimum of eighty days before the first counts were taken. During the next four or five months, with continued treatment at this level, the total white blood counts did not change significantly except when thyrotrophic hormone was administered to two of the animals, when an increase of considerable magnitude occurred.

In two of the monkeys (694 and 788) a significant granulocytopenia appeared (Table 1). A low value of 9% neutrophiles in a total of 3500 white blood cells was observed on one occasion (2-2-45). After six weeks of observation had shown that the granulocytopenia in these two animals was fairly constant, each was given six daily intravenous injections of 100 milligrams of pyridoxine, along with the usual 0.8 gm. per day of thiouracil. No noteworthy alteration in either total white blood cell count or neutrophile percentage followed this treatment. Five weeks later, while continuing the thiouracil regimen, folic acid was given orally to each animal, 1.8 mgms. daily for 12 days. This agent, too, failed to affect significantly the blood picture when given concurrently with thiouracil. The percentage of polymorphonuclear leucocytes was more than doubled when the foreign protein mixture containing the thyrotrophic hormone was injected.

Disturbances in Menstrual Rhythm

Hypothyroidism in female monkeys as well as in women is frequently reflected in menstrual irregularity. Hypothyroid monkeys have long periods of amenorrhea and are refractory to standard dosages of estrogens which normally result in estrogen withdrawal bleeding (Engle, 1944).

The menstrual cycles of the four monkeys treated with thiouracil are of interest in this respect. Monkeys in this laboratory have a menstrual rhythm in which the irregularity is not great. In this study an interval of 50 or more days between menstrual periods was arbitrarily designated as an indication of disturbed rhythm.

Monkey 784 showed considerable interruption of menstrual rhythm. Of ten menstrual periods during the treatment, one occurred after an intermenstrual interval of 69 days and one after a 127 day interval. Another animal with 9 menstrual periods, had 5 with an interval of more than 50 days between periods.

The animal with the least disturbance of rhythm during treatment was monkey 788, with 12 periods of which one was a period of

amenorrhea of 112 days. However, the rhythm was highly irregular, the cycles ranging between 21 and 38 days, with only 5 periods of statistically normal frequency.

The pattern of monkey 605 was characterized by very irregular menstrual cycles. Only 5 bleeding periods occurred during the 14 months of treatment, with but one of a normal length and including one interval of 186 days without menstruation.

General

None of the monkeys showed any striking change in degree of belligerence or of spontaneous activity. In one there was some suggestive appearance of fatty deposition around the neck region, but nothing resembling human myxedema was seen. The monkey does not show marked clinical evidence of hypothyroidism (Jailer et al. 1944).

SUMMARY

Thiouracil was administered to four adult female rhesus monkeys in increased doses up to 0.8 gm. each day for 14 months. Repeated studies were made of the white blood cells in the treated animals. In two of the animals a significant neutropenia developed, which was not improved by treatment with pyridoxine nor folic acid given during thiouracil administration. No significant changes in body weight or circulation time were observed during treatment. The serum cholesterol did not exhibit a significant trend during treatment, but it is possible that the thiouracil had a "labilizing" effect on the mechanism which controls the serum cholesterol. Menstrual irregularity was more pronounced during treatment, with frequent periods of amenorrhea.

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